Unravelling the therapeutic intervention of inflammation and cancer by Viscum album : understanding its anti-inflammatory and immunostimulatory properties

Chaitrali Saha

Par **Chaitrali SAHA**

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Thèse présentée
pour l'obtention du grade
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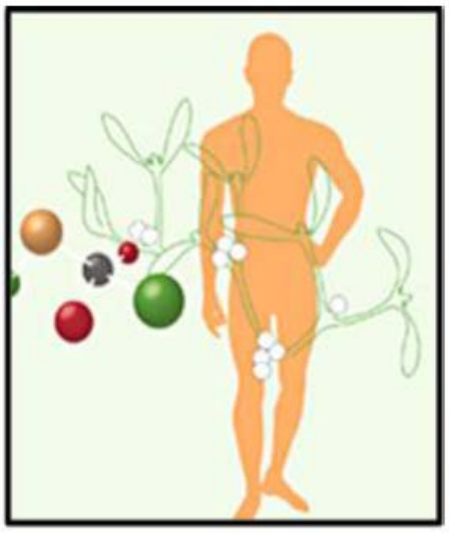

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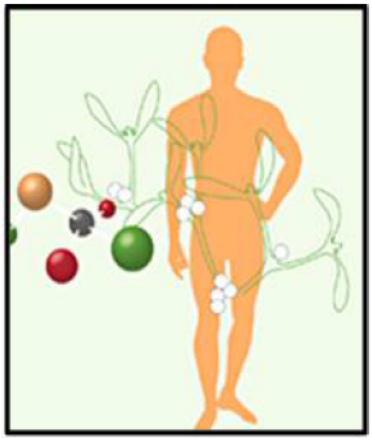
Sujet de la thèse

Etude des propriétés phytothérapeutiques de Viscum album dans le traitement de l'inflammation et du cancer: Détermination de ses caracteristiques anti-inflammatoires et d'immunostimulation



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"Take up one idea. Make that one idea your life - think of it, dream of it, live on that idea. Let the brain, muscles, nerves, every part of your body, be full of that idea, and just leave every other idea alone. This is the way to success." ***Swami Vivekananda***

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***This thesis is dedicated to my parents and my husband for their endless love, support and encouragement**.*

ABBREVIATIONS

AA	Arachidonic acid
Ab	Antibody
ADCC	Antibody-dependent cell-mediated cytotoxicity
AE	Adverse effect
Ag	Antigen
Apaf	Apoptosis-associated factor
APC	Antigen presenting cell
ARE	Adenylate-uridylate-rich element
Arg 1	Arginase 1
BAD	Bcl-2-associated death promoter
BAX	Bcl-2-associated X protein
BCL	B-cell lymphoma
CAM	Complementary and alternative medicine
CD	Cluster of differentiation
COX	Cyclo-oxygenase
COXIB	COX-2 inhibitor
CRF	Cancer-related fatigue
CTL	Cytotoxic T lymphocyte
DC	Dendritic cell
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
EGF	Epidermal growth factor
ELISA	Enzyme link immunosorbent assay
ERK	Extracellular-signal-regulated kinase
FAP	Familial adenomatous polyposis
FCS	Foetal Calf Serum
FcγR	Fc gamma receptor
FITC	Fluorescein isothiocyanate
Foxp3	Forkhead box P3
GMCSF	Granulocyte-macrophage colony-stimulating factor
HUVEC	Human umbilical vein endothelial cell
IFN	Interferron

Ig	Immunoglobulin
IgE	Immunoglobulin E
IL	Interleukine
IRF	Interferon regulatory factor
IVEC	Immortalised human venous endothelial cell
JNK	c-Jun N-terminal kinase
LPS	Lipopolysaccharides
MAPK	Mitogen-activated protein kinase
MCSF	Macrophage colony-stimulating factor
MHC	Major Histocompability complex
miRNA	microRNA
ML	Mistletoe lectin
MMP	Mitochondrial membrane potential
MO	Monocyte
mRNA	Messenger RNA
MΦ	Macrophage
NEMO	NF-κB essential modulator
NF-κB	Nuclear factor kappa B
NK	Natural killer cells
NLR	Nucleotide-binding oligomerization-domain protein like receptor
NOD	Nucleotide-binding oligomerization-domain protein
NOS	Nitric oxide synthase
NSAID	Non-steroidal anti-inflammatory drugs
PAMP	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffer saline
PG	Prostaglandin
PRR	Pathogen- recognition receptor
PTGS	Prostaglandin-endoperoxide synthase
QOL	Quality of life
RNA	Ribonucleic acid
RNI	Reactive nitrogen intermediates
RNS	Reactive nitrogen species
ROI	Reactive oxygen intermediates

ROR-γt/ RORC	Retionic acid-related orphan receptor
ROS	Reactive oxygen species
RIP	Ribosome inactivating protein
rRNA	Ribosomal ribonucleic acid
SEM	Standard error of mean
STAT	Signal transducer and activator of transcription
TAA	Tumor-associated antigen
TAM	Tumor associated macrophage
T-bet	T box transcription factor
TFH	Follicular helper T cell
TGF-β	Transforming growth factor β
Th	Helper T cells
TLR	Toll-like receptor
TNF	Tumor necrosis factor
Tregs	Regulatory T cells
UTR	Untranslated region
VA	*Viscum album*
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A

Résumé en Français

Etude des propriétés phytothérapeutiques de *Viscum album* dans le traitement de l'inflammation et du cancer: Détermination de ses caractéristiques anti-inflammatoires et d'immunostimulation

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<< Biotechnologie et mise en œuvre des Fonctions Biologiques >>

Thèse est présentée à la Faculté de l'Université de Technologie de Compiègne

en vue l'obtention du grades de

Philosophiae Docteur (Ph.D.) de l'Université de Technologie de Compiègne

Les mots clés : Viscum album, lectine de gui, cyclo-oxygénases, PGE2, effet anti-inflammatoire, effet immunomodulateur,

Les préparations de *Viscum album* (VA), connu sous le nom vernaculaire de gui européen, sont fréquemment utilisées en support des traitements anticancéreux, principalement pour améliorer la qualité de vie des malades et réduire la croissance des tumeurs. Elles sont connues pour exercer des effets anti-tumoraux. Il existe de plus en plus de données scientifiques faisant état de liens étroits entre cancer et inflammation. Etant donné que la prostaglandine E2 (PGE2) induite par la cyclo-oxygénase 2 (COX-2) joue un rôle clef dans l'inflammation, j'ai exploré la régulation du système COX-2-PGE2 par VA et ses mécanismes sous-jacents. J'ai montré que VA exerce ses effets anti-inflammatoires en inhibant sélectivement l'expression de COX-2 et en diminuant la production de PGE2 qui en découle, par le biais d'une déstabilisation de l'ARNm de COX-2. En plus de leurs propriétés cytotoxiques, il a été montré que les préparations de VA ont également des effets immunostimulants. Les différentes préparations de VA sont hautement hétérogènes du fait de leurs compositions biochimiques qui varient selon la récolte, l'espèce de l'arbre hôte et les méthodes de préparation qui peuvent influer sur leur efficacité clinique. De ce fait, j'ai réalisé une étude comparative sur cinq préparations de VA dans le but d'analyser leurs capacités de maturation et d'activation des cellules dendritiques (DC) qui peuvent à leur tour présenter une réponse immunitaire anti-tumorale. Les résultats ont montré que parmi les cinq préparations, VA Qu Spez induit de manière significative l'activation des DC et la sécrétion de cytokines pro-inflammatoires telle que l'IL-6, l'IL-8 et le TNF-α qui induisent la production d'IFN-γ, orientant de ce fait la réponse immunitaire vers une réponse Th1. L'orchestration de la

fonction des cellules myelomonocytiques est un élément central à l'interface entre inflammation et cancer. Il constitue un paradigme expliquant la plasticité et la fonction des macrophages. Mon étude met en évidence l'influence de VA Qu Spez sur la polarisation des macrophages qui passent d'un état alternatif (M2) à un état dit classique (ou M1). Les macrophages M2 sont connus pour polariser les réponses immunitaires Th2, pour participer à l'élimination des parasites, pour diminuer l'inflammation, pour promouvoir le remodelage tissulaire et la progression des tumeurs et pour avoir des fonctions immunorégulatrices. Les macrophages M1 sont impliqués dans la réponse Th1, favorisent la résistance aux pathogènes intracellulaires et aux tumeurs et promeuvent des réactions de désagrégation tissulaires. L'ensemble de ces résultats permet de comprendre les propriétés anti-inflammatoires et immunostimulantes des préparations de VA. Des recherches complémentaires permettront d'améliorer les stratégies d'utilisation thérapeutique de VA et son utilisation dans les soins de support aux traitements anticancéreux.

Abstract in English

Unravelling the therapeutic intervention of inflammation and cancer by *Viscum album*: Understanding its anti-inflammatory and immunostimulatory properties

by

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Thesis is presented at the Faculty of Université de Technologie de Compiègne
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Key words: Viscum album, mistletoe lectin, cyclo-oxygenases, PGE2, anti-inflammatory effect, immunomodulatory effect, complementary and alternative medicine.

Viscum album (VA) preparations, commonly known as European mistletoe, are frequently used as complementary therapy in cancer, mainly to improve quality of life of the patients and to reduce the tumor growth. They are known to exert anti-tumoral effects. There is increasing evidence of the convoluted connection of cancer and inflammation. As cyclooxygenase-2 (COX-2)-induced prostaglandin E2 (PGE2) plays a key role in the inflammation, I explored the regulation of COX-2-PGE2 axis by VA and underlying mechanisms. I found that VA exerts anti-inflammatory effects by selectively inhibiting COX-2 expression and ensuing PGE2 production. Inhibition of COX-2 expression implicates COX-2 mRNA destabilisation. In addition to their cytotoxic properties, they have also been shown to have immunostimulatory properties. Each VA preparations are highly heterogeneous because of their chemical composition which varies depending on the time of harvest, species of host tree and manufacturing methods, together which might influence clinical efficacy of VA. Therefore I performed a comparative study involving five different preparations of VA concerning maturation and activation of dendritic cells (DCs) which in turn may manifest anti-tumoral immune response. Results showed that among all five preparations, VA Qu Spez significantly induces DC activation, secretion of pro-inflammatory cytokines such as IL-6, Il-8 and TNF-α, enhancing IFN-γ production hence promoting Th1 immune response. The orchestration of myelomonocytic cell function is a key element that links inflammation and cancer and provides a paradigm for macrophage plasticity and function. My study reveals the effect of VA Qu Spez in switching the M2 macrophages which are known to participate in polarizing Th2 responses, help with parasite clearance, dampen inflammation, promote tissue remodelling and tumor progression and have immunoregulatory functions, towards classically activated M1 macrophages which are part of a polarized Th1 response and mediate resistance

to intracellular pathogens and tumors and elicit tissue-disruptive reactions. These results together should assist in understanding the anti-inflammatory and immunostimulatory properties of VA preparations and further research is warranted to improve the therapeutic strategies of use of VA and their role as complimentary therapy in cancer.

INTRODUCTION

The Immune System

The immune system is an organization of cells and molecules with specialized functions. Immunologic defences in vertebrates comprise of two fundamentally different types of responses to invading microbes. Natural or innate responses occur to the same extent even after encountering the infectious agent for several times, whereas acquired or adaptive immune responses improve upon exposure to a given infection repeatedly. Today these two types of immune responses are well appreciated as obligatory part of immune system mediating successful immune responses towards infection and tissue injury. The innate immunity encompasses the elements of immune system which includes phagocytic cells such as neutrophils, monocytes, macrophages, cells which release inflammatory mediators such as basophils, mast cells, eosinophils and natural killer cells for immediate host defence. The molecular components are complement, cytokines and acute phase proteins. Adaptive immunity is triggered when B and T cell receptors encounter antigens and lead to proliferation of these antigen-specific cells. With the help of T cells, B cells secrete antigen specific immunoglobulins, and then activate macrophages to eliminate intracellular pathogens. The innate response hampers normal tissues due to lack of specificity but the process is rapid, whereas the adaptive immunity can be precise and flexible but can take several weeks to develop and is able to combat the infections which evade the innate immune responses (Janeway and Medzhitov 2002). Immune cells are generated from pluripotent stem cells in the fetal liver and bone marrow and then circulate throughout the extracellular fluid in the body. Within the bone marrow B cells mature, but for T cells they have to travel to the thymus to mature.

Rapid response: Innate Immune System

The innate immune system is all about the immune defence which lack immunologic memory. Thus the main characteristic of this kind of immune system is that it remains unaltered even after several times of interactions with the antigen. It is believed that these kinds of responses developed earlier in evolution than acquired responses (Delves and Roitt 2000). The cellular components of the innate immune response are dendritic cells, monocytes, macrophages, granulocytes, natural killer cells, even the skin, pulmonary and the gut epithelial cells that form the interface between an organism and its specific environment. The non-cellular aspects of innate immune system includes complement cascade, which is specialised to prevent the entry of pathogens through physical blockade, or destroying the pathogens directly bringing them to the attention of phagocytes (Clark and Kupper 2005). The immune system has evolved to recognise pathogen-associated molecular patterns (PAMP)

common to diverse class of pathogens. PAMPs includes lipopolysaccharides (LPS), aldehyde-derivatized proteins, denatured DNA, mannans, teichoic acids and bacterial DNA (Medzhitov and Janeway 2002). The PAMPS are recognised by conserved proteins pathogen- recognition receptors (PRRs) (Janeway and Medzhitov 2002). PRR mediates many steps in inflammation which includes phagocytosis, activation of signalling pathways in inflammation, induction of cell death, activation of complement cascades etc. Another important pathogen recognition receptor is Toll-like receptor (TLR), expressed on innate immune cells, on endothelial cells, epithelial cells and fibroblasts (Janeway and Medzhitov 2002), (Schnare, Barton et al. 2001). Phagocytes are activated when TLR interacts to their microbial ligands, leading to direct killing of pathogens and secrete pro-inflammatory cytokines and anti-microbial peptides (Takeda, Kaisho et al. 2003). In addition, these TLRs activate dendritic cells (DCs), thus play important role in initiation of adaptive immunity. TLRs trigger NF-κB signalling pathway, which masters the switch for induction of inflammation pathway (Takeda, Kaisho et al. 2003). Other tissue factors include heat-shock proteins, cytokines, chemokines, extra-cellular matrix components, lectins, lipids etc. lead to phagocyte and DC activation which in turn initiates adaptive immune response. There are additional components of innate immune system; these are anti-microbial proteins such as large proteins like lysozyme and cathepsin G, smaller peptides like cathelicidins, defensins, and skin-antimicrobials like dermcidin and psoriasin (Ganz 2003), (Madsen, Rasmussen et al. 1991), (Schittek, Hipfel et al. 2001). A central feature of innate response is neutrophil recruitment and activation at the infection site to eradicate the pathogen (Witko-Sarsat, Rieu et al. 2000). Blood-borne monocyte derived macrophages possess receptors like mannose for carbohydrates that are not exposed on vertebrate cells, thus become able to discriminate between “foreign” and “self”. Macrophages and neutrophils both contain receptors for complement and antibodies, thus enhance phagocytosis (Aderem and Underhill 1999). A key cellular component of innate immunity is the dendritic cells, the cells which are constantly involved in the endocytosis of the extracellular antigens (Bell, Young et al. 1999). Unlike macrophages and neutrophils, eosinophils are the only phagocytic cells which are weak in their effect, however only on activation they secrete reactive oxygen metabolites and cationic proteins to kill parasites (Wardlaw, Moqbel et al. 1995). Basophils and mast cells contains high affinity IgE receptors (FcεR) (Kinet 1999). In atopic allergies such as asthma, hay fever, eczema, an allergen bind to IgE and cross-links to FcεR and this process further triggers specialized cells which release inflammatory mediators such as prostaglandins, histamine and leukotrienes. Natural killer cells (NK) remove infected cells in one of the two ways (Biron, Nguyen et al. 1999). First, the Fc receptors link NK cells to IgG-coated target cells, and the target cells are destroyed by

antibody-dependent cellular cytotoxicity. Second, the killer-activating receptors of NK cells recognize different molecules present on all nucleated cells, whereas the killer-inhibitory receptors recognize MHC-I present on the surface of all nucleated cells. When the killer-activating receptors are blocked, the instruction of killing by NK cells is overruled by an inhibitory signal (Moretta, Biassoni et al. 1997), (Lanier 1998).

Adaptable but Dependent Response: Adaptive Immune System

T and B lymphocytes are the cellular components of adaptive immune system. Flexibility and memory are the hallmarks of this acquired immune response. T and B cells involve recombination of antigen receptor genes to create unique antigen receptors which recognize virtually any antigen, unlike the innate cells. The memory of this system is novel as the B and T cells are capable of retaining the encountered antigen for a long time within an organism and provide rapid responses to reinfection. The antigen receptors on B cells are the antibodies encoded by the heavy and light immunoglobulin (Ig) genes. Antibodies are classified based on the isotype of their heavy chains; they are IgM, IgG, IgE. Initially B cells produce pentameric IgM, however with the influence of T cell cytokines; B cells undergo isotype switching and generate IgG subtypes, IgE or IgA. T cell receptors are never secreted and T cells recognize peptides which are generated by proteolytic cleavage of antigens. Thus T cells recognize the primary structure of a protein, whereas B cells recognize the tertiary structure. The unique feature of T cells is that they can only recognize antigenic peptides when they are bound to major histocompatibility complex (MHC)-I/ MHC-II proteins. Cellular cross-talk is necessary for adaptive immune response. In response to an antigen, naïve B cells are stimulated by $CD4^+$ helper T cells, followed by their proliferation and differentiation. T cells require a second signal for their proliferation and differentiation. B and T cells orchestrate the adaptive immune response engaging them in a complex dialog. Based on the specific functions and migration patterns T cells can be divided into distinct subsets. With the expression of the homing receptors L-selectin and CCR7, it is observed that naïve T cells recirculate between blood and lymph nodes primarily (Mackay, Marston et al. 1990), (Sallusto, Lenig et al. 1999). Memory T cells can be again divided into other two subsets, namely central memory T cells and effector memory T cells (Sallusto, Geginat et al. 2004). Central memory cells have long lived memory and they primarily travel between blood and lymph nodes, may also migrate to peripheral tissues (Campbell, Murphy et al. 2001). In contrast, effector memory T cells have short life and they are aggressive in nature, migrate to the target tissues and finally neutralize the pathogen (Sallusto, Geginat et al. 2004). $CD4^+$ T helper cells comprises of several subtypes, such as Th1, Th-2 and T-regulatory cells (Treg).

Th-1 secretes IFN-γ and TNF-β, and is able to activate macrophages and stimulate cytotoxic T lymphocytes, thus induces cell-mediated immune response. Th2 cells secrete interleukins such as IL-4, IL-5, IL-13 and is capable of activating B cells to generate antibodies, specifically IgE, thus induces humoral immune response (Mosmann and Coffman 1989). The immune response can be cellular or humoral based on the specific response towards the pathogen. Th1 promotes cellular immunity whereas Th2 promotes humoral immunity. In some exceptional Th2 autoimmune diseases like lupus, it is found that IFN-γ (Th1 cytokine) induces B cell production of IgG2a antibodies (Gavalchin, Seder et al. 1987), (Snapper and Paul 1987). Tregs are responsible for self-tolerance, but may interfere with tumor immunity (Sakaguchi, Sakaguchi et al. 2001). T cell polarization regulates the adaptive immunity.

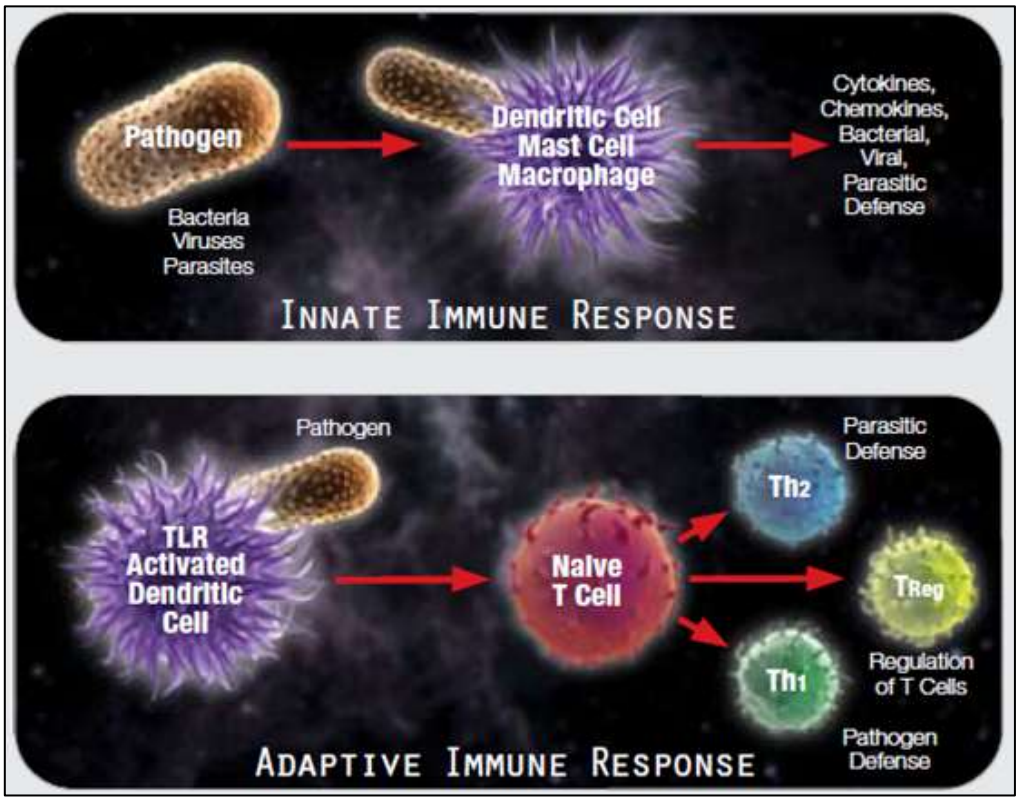

Figure 1: The three sentinel cells, Dendritic, Mast, and Macrophages serves protection against ingested pathogens.

The Bridge between Old and New: Dendritic cells the Key Players

Dendritic cells are the central players of the immune system and they have the ability to stimulate naïve T cells to respond to antigen (Banchereau and Steinman 1998).Dendritic cells are capable of loading endocytosed antigenic peptides on both MHC class I and MHC class II molecules, and present them to CD8 and CD4 T cells (Rescigno, Citterio et al. 1998), (Guermonprez, Saveanu et al. 2003). They develop in the bone marrow and travel to the

tissues in an immature state. Dendritic cells undergo maturation when they encounter a number of danger signals including PAMP, cytokines and tissue factors (Chain 2003). These mature dendritic cells are extremely potent activators of T cells and their response. Dendritic cells pass a sequence of signals to responding T cells. The initial signal is the interaction of the T cell receptors to the specific antigen and MHC on the surface of dendritic cells and decides the specificity of the antigen to response. The next signal is the co-signaling which is required for T cells to decide to respond to the antigen. The co-signaling is of two types, co-stimulation or co-inhibition, which is generally provided by the growing dendritic cells and these cells are able to promote a Th1 type of T cells in both mice and humans (Langenkamp, Messi et al. 2000), (Boonstra, Asselin-Paturel et al. 2003). The binding of mycoplasma derived lipopeptide 2 to TLR-2 (TLR-6 heterodimers) induces dendritic cells to secrete IL-10 but not IL-12 and these dendritic cells encourage unpolarized T cell responses (Weigt, Muhlradt et al. 2003). Schistosomamansoni secretes lysophosphatidylserine which triggers TLR2 which in turn stimulates dendritic cells. These stimulated dendritic cells induce T cells to adopt a Treg population by secreting IL-10 which is a well-known regulatory cytokine (van der Kleij, Latz et al. 2002). The Th2-biased host response arise from the ability of parasitic antigens to induce development of type 2 dendritic cells. A glycoprotein, Es-62, from Acanthocheilonema, induces formation of dendritic cells of type 2 and in turn helps in the development of Th2 T cells (Whelan, Harnett et al. 2000). An increasing number of pathogens are being identified which induce regulatory dendritic cells and thus induce formation of regulatory T-cell responses. Bordatella pertussis- hemagglutinin and S.mansoni-lysophosphatidylserine efficiently generates regulatory dendritic cells hence developing regulatory T cells (McGuirk, McCann et al. 2002), (van der Kleij, Latz et al. 2002). When T cells are primed by activated dendritic cells situated in the gut-associated lymph nodes an up regulation of gut-homing adhesion molecules takes place which preferentially send them back to this tissue in future (Campbell and Butcher 2002). In summary, dendritic cells provide three additional signals to T cells which fine tune the immune response. Innate immune response is vital to initiate T cell response and this type of immune response has ability to modulate tolerance of T cells to antigens. The dendritic cells which have not received any danger signal present antigen to T cells in the absence of co-signaling molecules. This kind of T cells can be functionally silenced and become unresponsive to antigens in future (Baxter and Hodgkin 2002), (Chen 2004). Dendritic cells are able to form Treg response which is the immune suppressive response. Lastly, the innate immune system can hamper tolerance by withdrawing the action of Tregs. Thus the innate immune system can induce or suppress

tolerance. Dendritic cells are the key players which can link two the innate and the adaptive immune responses.

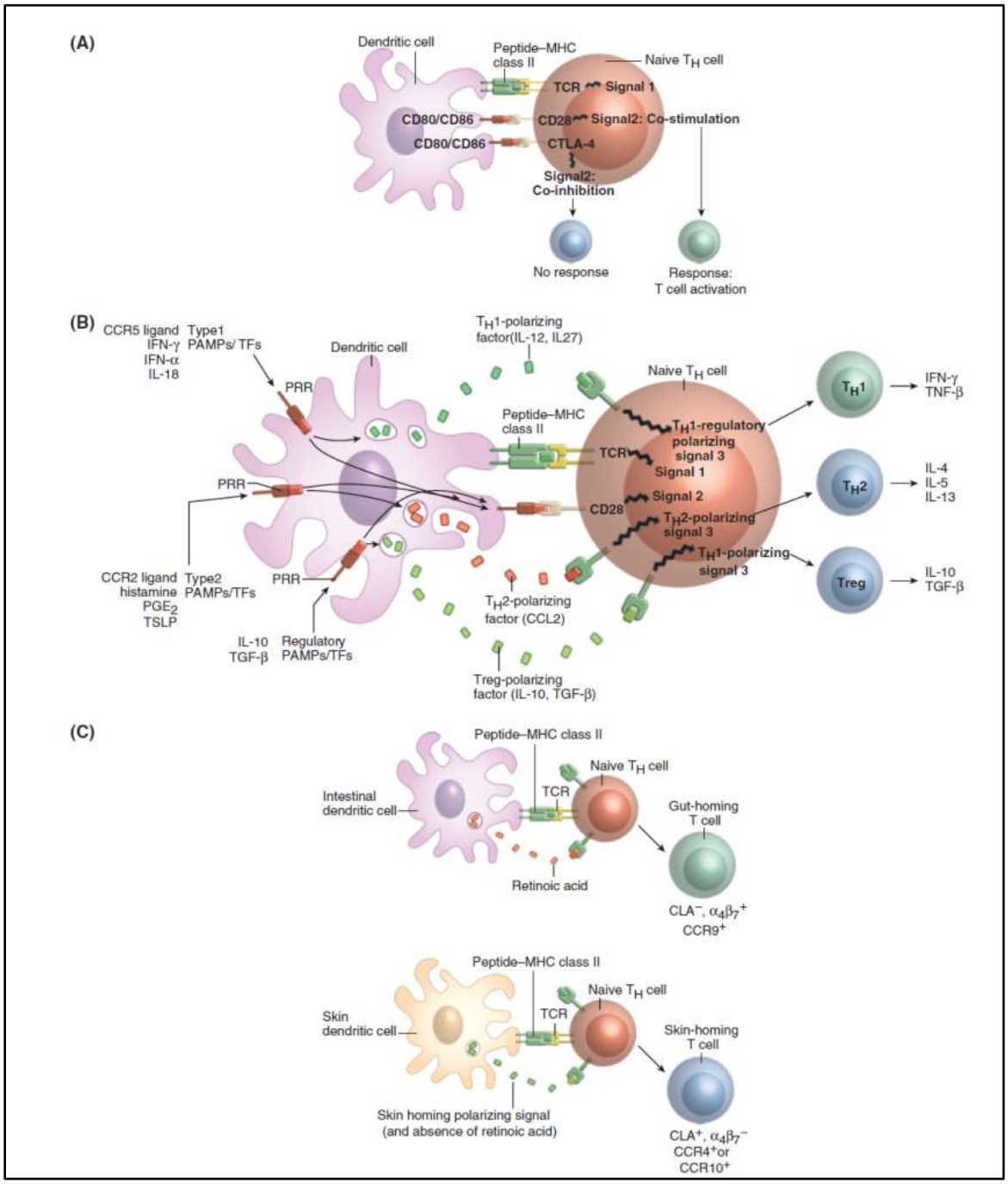

Figure 2: Dendritic cells: Bridge between old and new. (A) Signal one determines antigen specificity and consists of interaction of the T cell receptor (TCR) with peptides loaded onto dendritic cell major histocompatibility (MHC) molecules. Signal two consists of co-signaling and can be either positive, leading to cell activation (co-stimulation) or negative, leading to no response (co-inhibition). (B) Signal three involves the polarization of CD4 T cells into Th1, Th2, or regulatory T cells. In general, viral-associated PAMP give rise to Th1 responses, and

PAMP from parasitic organisms favour Th2 responses. (C) Signal four leads to spatial imprinting of T cells, leading to the acquisition of homing receptors that induce selective recirculation through the tissue in which antigen was first encountered. (Adapted from Clark R, J invest Dermatol, 2005)

Macrophage Biology in Homeostasis and Disease: Full Spectrum of Macrophage Activation

The orchestration of myelomonocytic cell function is a major step that connects inflammation and cancer and sets up a paradigm for macrophage plasticity and function (Biswas and Mantovani 2010). One of the hallmarks of adaptive immunity is their ability to mount an enhanced immune response after the re-exposure of the same antigen. Similarly, sensing of ingested microorganisms by macrophage cells results in their functional stimulation, thus in response to phagocytes innate immune system generates a response which follows by a built-in adaptive response (Bowdish, Loffredo et al. 2007), (Mantovani 2008). Encountering microbial components such as lipopolysaccharide (LPS) is known to be a potent activator of macrophages (Gordon and Taylor 2005). In response to microbe recognition, macrophages produce huge amount of fluid-phase pattern-recognition molecules known as ante- antibodies (Bottazzi, Doni et al. 2010). The repertoire of fluid-phase patter-recognition molecules includes molecules which belong to ficolin family, collectin family and pentraxin family. Pentraxin 3 is characterised to be responsible for the interaction of cellular and humoral arms of innate immunity (Jeannin, Bottazzi et al. 2005).

Considering the Th1 and Th2 polarization, two distinct subtypes of polarized macrophages are identified: the classically activated macrophages-M1 and the alternatively activated macrophages-M2 (Gordon and Taylor 2005), (Mantovani, Sozzani et al. 2002). IFN-γ or LPS polarize macrophages towards M1, whereas M2 polarization was discovered as an original response to Th-2 cytokine IL-4 (Stein, Keshav et al. 1992). M2 macrophages are prone to phagocytosis. They are characterized by high expression of scavenging, mannose and galactose receptors. Through arginase pathway they produce ornithine and polyamines. M2 macrophages express high level of IL-10 and low level of IL-12 (Gordon and Taylor 2005), (Mantovani, Sozzani et al. 2002), (Mantovani 2008). These M2 phenotypic macrophages polarize Th2 response, suppress immune response, dampen inflammation, clear pathogens, promote tissue remodelling, and supports tumor growth. In contrast, M1 macrophages exerts cytotoxic effect towards ingested microorganisms and cancer cells, thus characterized as the professional antigen presenting cells enhancing immune response and promoting tumor regression. M1 and M2 macrophages have distinct functions and chemokine profile. M1 expresses CXCL9 and CXCL10 which are Th1 cell-attracting chemokines, whereas M2

expresses CCL17, CCL22, CCL24 (Martinez, Gordon et al. 2006). Th1 cells can drive M1 macrophage polarization by producing IFN-γ. The M1 cells release large amount of pro-inflammatory cytokines such as IL-12, IL-23 and tumor necrosis factor (TNF). They are characterized by higher expression of MHCII, co-stimulatory molecules, secretion of reactive nitrogen intermediates, reactive oxygen species, elevated capacity of antigen presentation and tumoricidal activity (Gordon and Taylor 2005), (Mantovani 2008). M1 macrophages through their secreting cytokines and chemokines recruit Th1 cells, amplify them and promote a Th1 immune response. Th2 cell-derived IL-4 and IL-13 directs M2 polarization during helminth infection (Loke, Nair et al. 2002), (Raes, Brys et al. 2005). M2 macrophages support angiogenesis and lymph agiogenesis by releasing several pro-angiogenic factors like EGF, VEGFA, VEGFC and IL-8 (Mantovani, Sozzani et al. 2002), (Schmidt and Carmeliet 2010), (Lin, Li et al. 2006), (Murdoch, Muthana et al. 2008). Macrophages act as „cellular chaperones" which help in the fusion of endothelial tip cells and participate in vascular sprouting (Fantin, Vieira et al. 2010), (Schmidt and Carmeliet 2010).

Cancer serves as a major paradigm of macrophage diversity and plasticity (Mantovani, Sozzani et al. 2002), (Biswas, Sica et al. 2008), (Lewis and Pollard 2006). Macrophages from metastatic mouse and human tumors are of M2 phenotype (Pucci, Venneri et al. 2009), (Sica and Bronte 2007). In the tumor microenvironment, macrophages are characterized by shoeing low level of IL-12 and high level of IL-10, show impaired expression of nitrogen and oxygen intermediates, low antigen presentation and tumoricidal activity, and inducing expression of several angiogenic component (Biswas, Gangi et al. 2006), (Hagemann, Lawrence et al. 2008), (Torroella-Kouri, Silvera et al. 2009), (Sierra, Corso et al. 2008), (Loges, Schmidt et al. 2010). Tumor cells entertain their interactions with macrophages by escaping phagocytosis (Jaiswal, Jamieson et al. 2009) and by encouraging M2 polarization via chemokines and cytokines such as CCL2 (Roca, Varsos et al. 2009), MSF, TNF, IL-10, TGF-β (Solinas, Schiarea et al. 2010), (Mantovani, Allavena et al. 2008), (Hagemann, Wilson et al. 2006). Strong genetic evidence suggests that Th2 derived IL-4 and IL-13 can activate M2 and their protumoral function. In mammary carcinoma, Th2 derived cytokines IL-4 and IL-13 induce polarization of tumor associated macrophages (TAMs) M2, hence promote tumor growth. In contrast it has been shown that blocking of IL-4 or IL-4Rα diminishes lung metastasis, which correlates with lower expression of *Arg 1* and *Tgfb* 1 (M2 genes) but higher expression of IL-6, NOS2, IL12a (M1 genes) by TAM"s. The M2 pro-tumoral phenotype of TAMs in cancer is reversible (Saccani, Schioppa et al. 2006), (Stout, Watkins et al. 2009). IFN-γ abrogates level of TAMs *in vitro* (Duluc, Corvaisier et al. 2009). M1 or the classically activated macrophages eliminate cancer cells and elicit tumor destructive properties (Schmidt and Carmeliet

2010).Activation of TLR9 by its ligand CpG together with IL-10, switches TAMs from M2 towards M1 phenotype (Guiducci, Vicari et al. 2005). Notch signalling in macrophages supports anti-tumor activity by promoting M1 phenotype (Wang, He et al. 2010). TAM infiltration is a favourable prognostic indicator of classical macrophage activation (Galon, Costes et al. 2006). The ability of macrophages to profoundly reprogram their functions smudges the difference between innate and adaptive response.

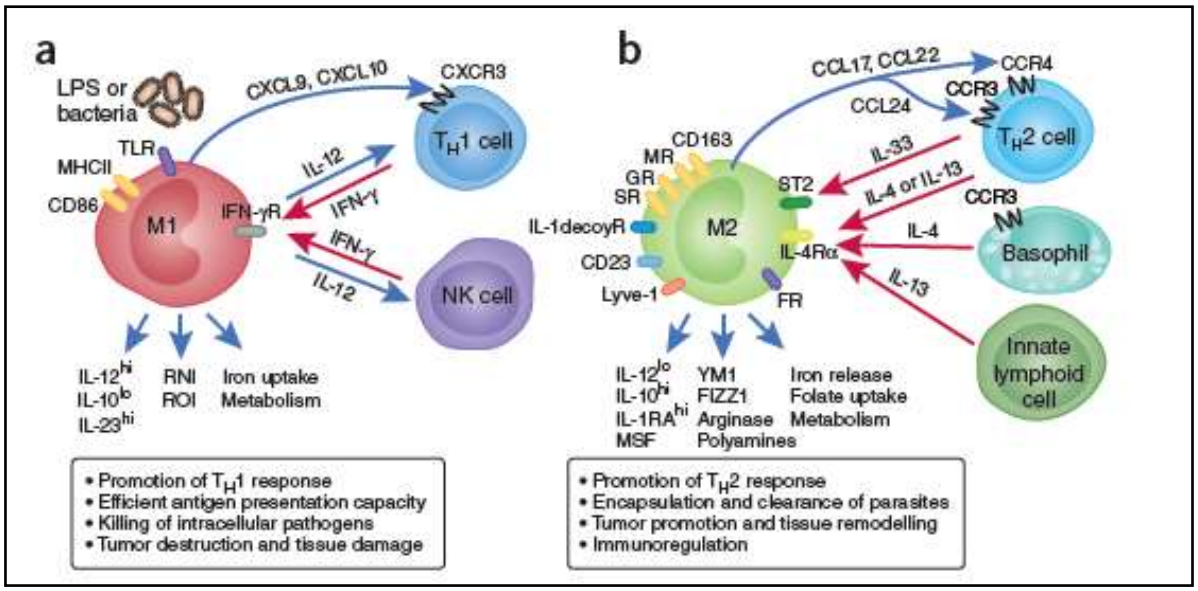

Figure 3: The orchestration of macrophage activation and polarization by lymphoid cells. (a) M1-polarized macrophages and their crosstalk with Th1 and NK cells. (b) M2 polarization of macrophages driven by Th2 cells, basophils and innate lymphoid cells through their secretion of IL-4, IL-13 or IL-33. (Adapted from Biswas, S. K., Nat Immunol, 2010)

T cell Polarization and Th cell Subsets

Human $CD4^+$ T cells are critical regulators of immune system. $CD4^+$ T cells are highly heterogeneous in human adults as they are generated in response to different pathogens and they are increasing in number of various subsets with specialized functions (Geginat, Paroni et al. 2013). Helper T cells are defined on basis of the cytokines and/or the expression of characteristic lineage-defining transcription factors. Five principal subsets of $CD4^+$ T cells have been identified so far: T helper (Th) 1, Th2 and Th17 cells which are specialized pathogen targeting cells (Mosmann and Coffman 1989), (Romagnani 1997), (Korn, Oukka et al. 2007), regulatory T cells (Treg) involved in self-tolerance (Sakaguchi 2005) and the follicular helper T cells (T_{FH}) which help in antibody production along with B cells (Crotty 2011). As naïve T cells have stem cells like properties, they can differentiate into virtually

any type of the above mentioned effector, memory or regulatory cells (Geginat, Paroni et al. 2014).

Th1 and Th2 effector T cells: The Tip of the Iceberg

Dendritic cells by producing IL-12, activate uncommitted naïve T cells, (Shortman and Heath 2010), (Nizzoli, Krietsch et al. 2013) which upon activation shows IFN-γ producing capacity. These types of T cells are called as Th1 which are induced upon encounter of intracellular pathogens like bacteria or viruses and are able to activate macrophages to eliminate intracellular bacteria. In contrast, in presence of IL-4, naïve T cells are primed to produce several cytokines like IL-4, IL-5, IL-10 and IL-13, but not IFN-γ. These types of T cells are called as Th2 cells and fight against extracellular parasites like helminths, and also involved in allergies (Robinson, Hamid et al. 1992). The capacity to produce either IFN-γ or IL-4 is permanently imprinted by epigenetic modifications like DNA methylation and histone acetylation (Kanno, Vahedi et al. 2012), (Allan, Zueva et al. 2012). The "master" transcription factors T-bet and GATA-3 are involved in generating Th1 and Th2 cells and they inhibit differentiation of alternative lineage.

FOXP3$^+$ Treg cells

These cells are required for self-tolerance. They were first identified in mice (Sakaguchi, Sakaguchi et al. 1995), and then in humans (Stephens, Mottet et al. 2001). Foxp3 transcription factor is required for their production (Hori, Nomura et al. 2003), (Fontenot, Rasmussen et al. 2005). Natural/ thymic FoxP3 Tregs become regulatory upon maturation in the thymus (Sakaguchi, Ono et al. 2006), whereas the adaptive/peripheral FoxP3 Tregs can be matured with influence of TGF-β from mature CD4$^+$Th cells (Fantini, Becker et al. 2006), (Tran, Ramsey et al. 2007). In humans CD45RA$^+$CD25$^+$FoxP3$^+$ cells indicate bona fide „naïve" thus the thymus derived Tregs, whereas CD45RA$^-$CD25$^+$FoxP3$^+$ cells represent an antigen-experienced thymic/peripheral mixed Treg population (Hoffmann, Eder et al. 2006). Stability of FoxP3$^+$ cells is always debated (Hori 2011). Tissue microenvironment is responsible for Treg functions. Tregs are capable of suppressing the Th cell lineages in mice, which are characterized to induce several transcription factors (Sawant and Vignali 2014). STAT3 expression in Tregs is required to suppress Th17 cells (Chaudhry, Rudra et al. 2009), IRF4 to suppress Th2 (Zheng, Chaudhry et al. 2009) and BCL-6 to suppress T_{FH} (Linterman, Pierson et al. 2011), (Chung, Tanaka et al. 2011). Stimulation with IL-12 insists FoxP3 Tregs to produce T-bet and IFN-γ in turn controls Th1 responses (Kleinewietfeld and Hafler 2013), (Sawant and Vignali 2014). These cells have cytotoxic properties in tumor-draining lymph

nodes in mice (Boissonnas, Scholer-Dahirel et al. 2010) and are able to inhibit anti-tumor CTL responses (Antony, Piccirillo et al. 2005).

Th17 cells

They are characterized by an independent differentiation lineage (Park, Li et al. 2005), (Harrington, Hatton et al. 2005), (Park, Li et al. 2005) and they express the lineage-specific transcription factor ROR-γt in mice and RORC2 in humans (Ivanov, McKenzie et al. 2006), (Unutmaz 2009). Th17 cells are important to eliminate extracellular bacteria and fungi as it is reported that Th17 lacking patients suffer from unhittable infection with *Candida albicans* (C. albicans) and *Staphylococcus aureus* (Ma, Chew et al. 2008). It was known that IL-12p40 and IL-12Rβ1 hetero-dimerize with respective IL-12p35 and IL-12Rβ2 to induce Th1 responses, but later it was demonstrated that they can also hetero-dimerize with respective IL-23p19 and IL-23R to initiate Th17 cells (McKenzie, Kastelein et al. 2006). TGF-β indirectly favours Th17 cell differentiation by inhibiting Th1 development (Santarlasci, Maggi et al. 2009). In absence of TGF-β1 (Acosta-Rodriguez, Napolitani et al. 2007), (Cosmi, De Palma et al. 2008), (Ghoreschi, Laurence et al. 2010) or in presence of TGF-β3 in mice (Lee, Awasthi et al. 2012), pathogenic Th17 cells are produced which secrete both IL-17 and IFN-γ both. In this situation, Th1/Th17 cells co-express RORC2 and T-bet, are predominantly present in autoimmune patients and are specific for both Th1 and Th17-inducing pathogens (Zielinski, Mele et al. 2012), (Duhen and Campbell 2014). *Ex-vivo* isolated human Th17 cells possess stable epigenetic marks at cytokine and transcription factor loci (Cohen, Crome et al. 2011), suggesting *in vivo* generated human Th17 cells are stable. It was identified that a rare population of human T cells simultaneously produces IL-4 and IL-17 (Cosmi, Maggi et al. 2010) and these cells were highly pro-inflammatory in allergic asthma. Th17 cells are highly heterogeneous and characterized by their ability to produce various effector cytokines such as Il-22 which promotes epithelial proliferation and barrier function (Zenewicz and Flavell 2008), IL-26 which is a pro-inflammatory cytokine and not expressed in mice (Dambacher, Beigel et al. 2009), IL-21 which inhibits GM-CSF and IFN-γ and promotes IL-10 secretion in developing Th17 cells (Peters, Lee et al. 2011).

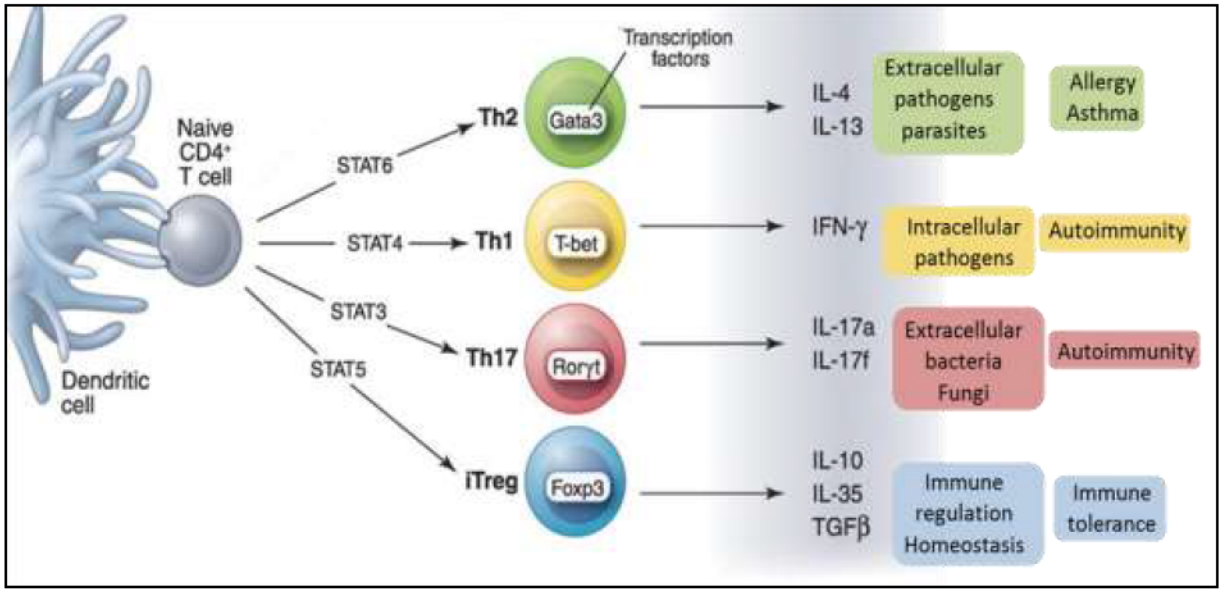

Figure 4: CD4$^+$ T cell differentiation. Naïve T cells upon activation can differentiate into specific lineage based on the cytokine milieu in the local environment. IL-12/IFN-γ promotes Th1; IL-4/IL-2 promotes Th2; TGFβ, IL-6, IL-21, IL-23 promotes Th17 and TGF-β/IL-2 promotes Tregs. These T cell lineage express specific set of transcription factors and cytokines which are crucial for effector function in host defense as well as in immune-mediated disease. (Adapted from O'Shea and Paul, 2010, Science)

Immunologic Dysfunction

A specialized controlled protection against the invading pathogens and cancer is served by a co-ordinated crosstalk between multi-component systems of innate and adaptive immunity. Immune system accomplishes dual faceted mechanism to perform this regulatory function. One hand, it serves protection to our body by fighting against infection and malignancies while on the other it can be deceitful, in attacking self-tissues and cells to produce devastating pathologies, and even more dangerous fatal autoimmune diseases (Matzinger 1994). Thus, any kind of misdirected or inappropriate immune responses lead to a number of human diseases. A hyperactive or undesirable immune response can lead to various immunological disorders like allergy, autoimmune disease and graft rejection in transplantation while insufficient or deprived immune response can be associated with immunodeficiency, chronic infections or cancer. The concept of recognising and eliminating primary developing tumors by immune system in absence of external therapeutic intervention has existed for nearly 100 years. However, it has been difficult to validate this concept. An accumulation of mutational

and epigenetic changes leads to cellular transformation and tumor development which pre dominantly alters normal cell growth and survival pathways (Smyth, Dunn et al. 2006).

Cancer Despite. Immunosurveillance: Means of Immunoselection and Immunosubversion

Numerous innate and adaptive immune effector cells and molecule exert important roles in recognising and eliminating cancer cells and this phenomenon is known as immunosurveillance. But in some cases cancer cells escape such immunosurveillance by two processes, i.e. Immunoselection: outgrowth of poorly immunogenic tumor cell variants and Immunosubversion: subversion of immune system (Zitvogel, Tesniere et al. 2006). Cancer is a serious manifestation of misdirected immune system, which results in failure of recognising the transformed cells and their killing by immune attack. According to Hannahan and Weinberg, tumor is characterized by six hallmarks (Hanahan and Weinberg 2000) and conceptual progress in the last decade has added few more emerging hallmarks (Hanahan and Weinberg 2011). Together they are as follows: 1) sustaining proliferative signalling, 2) evading the growth suppressors, 3) avoiding immune destruction, 4) limitless growth potential, 5)promoting inflammation, 6) an unusual ability to invade surrounding tissues and metastasize to distant organs 7) ability to sustain angiogenesis, 8) genome instability and mutation, 9) resisting cell death, and 10) deregulating cellular energetic.

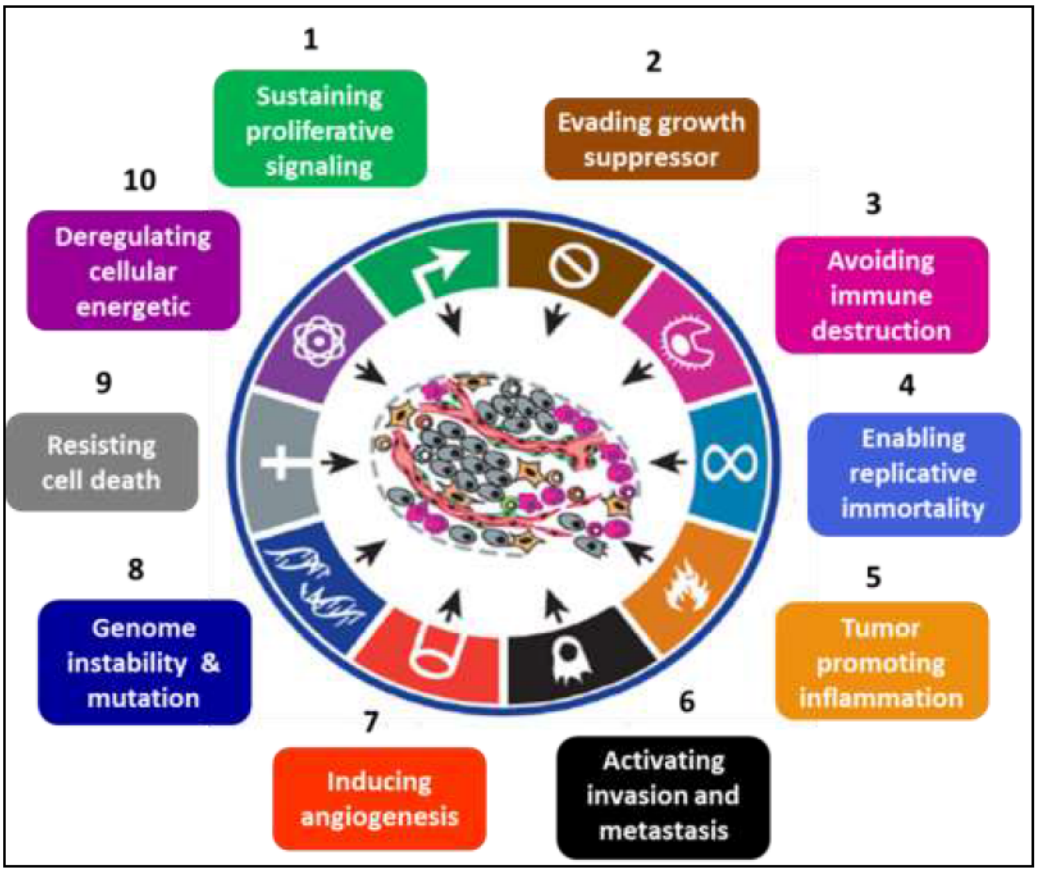

Figure 5: The hallmarks of cancer. (Adapted from Hanahan and Weinberg, Cell, 2011)

A key question in cancer immunology is whether recognition of tumor antigens by immune system leads to activation known as surveillance or tolerance. The fundamental processes of cancer progression are tissue invasion and metastasis, which are pro-inflammatory and thus activates innate and adaptive anti-tumor immunity (Pardoll 2003).

Immunosurveillance actively involves lymphocytes which act as sentinels in recognizing and eliminating continuously arising, nascent transformed cells (Shankaran, Ikeda et al. 2001). The fundamental of immune surveillance is that tumor arises with similar frequency to infection with pathogens and the immune system acts constantly by recognizing and destroying these tumors based on their expression of tumor-associated antigens (TAAs) (Pardoll 2003). Both spontaneously arising and chemically stimulated tumors show diverse properties, with some being rejected efficiently which are known as regressor-tumors and some progressively growing known as progessor-tumors. Cancer immunosurveillance appears to be an important host protection process that inhibits carcinogenesis and maintains regular cellular homeostasis. The recognitions that immune system plays a dual function in the complex interactions between tumors and the host defined a refinement of cancer immunesurveillance into "cancer immunoediting" (Dunn, Old et al. 2004). Gavin P Dunn

reported 3 Es of cancer immunoediting called elimination**,** equilibrium and escape. These effector immune cells employ extremely diverse mechanisms to control tumor targets including the induction of tumor cell death by mitochondrial and cell death receptor pathways. Tumors not only can survive and disseminate, but also, more importantly, they can mimic some of the signalling pathways of the immune system to propagate conditions that favour tumor immune tolerance thereby escaping the tumor immunity.

The immune response to tumors includes NK-cell activity, CTL-mediated lysis, macrophage-mediated tumor destruction, and ADCC mediated destruction (Smyth, Thia et al. 2000), (Girardi, Oppenheim et al. 2001). Several cytotoxic factors, including TNF-α, TNF-β and IFN-γ mediate tumor-cell killing (Kaplan, Shankaran et al. 1998). By modulating their tumor antigens, reducing expression of class I MHC molecules, and by antibody mediated or immune complex-mediated inhibition of CTL activity, tumors may evade the immune response. Both innate and adaptive immune systems play an important role in cancer immune editing. Recent investigate several models for the role of innate immunity in recognition and elimination of malignant cells, where innate immune cells can sense transformed cells through expression of molecules up-regulated during the process of malignant transformation and tumor progression (Vesely, Kershaw et al. 2011). With respect to the self and non-self paradigm, two types of receptors on innate immune cells namely toll-like receptors (TLR) and the NKG2D receptor play an important role. Toll-like receptors expressed by APCs recognize non-self-molecules, e.g., pathogen-associated molecular patterns (PAMPS) such as bacterial cell wall structures and viral polynucleotides. However NKG2D receptor of lymphocytes recognizes self-ligands expressed by cancer cells. The discovery of self-innate immune receptors that are involved in activation of the innate and adaptive immune system results in reconsideration of the framework of "evolution of an immune system to recognize foreign". T cells, NK cells, and NKT cells express NKG2D receptors (Diefenbach, Jensen et al. 2001). Ligands for NKG2D receptors include major histocompatibility complex (MHC) class I chain related (MIC) A and MIC B on human cells. Ligands that are induced only in the context of malignancy are not recognized by innate immune cells, but recognize ligands that are up-regulated by non-malignant cells during oxidative stress, heat shock, altered cell cycle regulation, and viral or bacterial infection (Zafirova, Wensveen et al. 2011).

Immune reactions can also potentially promote cancer development and growth. Chronic inflammatory responses, a feature of innate immunity, can contribute to the development of cancer. Additionally, the activation of immune cells places these cells at risk for cancer. For example, the activation B lymphocytes require various DNA modifying activities, errors in

which can result in molecular lesions (oncogene mutation, chromosomal translocations) that lead to cancer.

As there are „3E‟s suggested by Gavin P. Dunn, existing in cancer editing as follows E1: elimination, E2: equilibrium, E3: escape, there are even „3S‟s exists suggested by Weiping Zou, which includes 3 therapeutic strategies they are S1: subversion of tolerizing conditions S2: supplementation of immune elements and S3: suppression of tumor angiogenesis and growth.

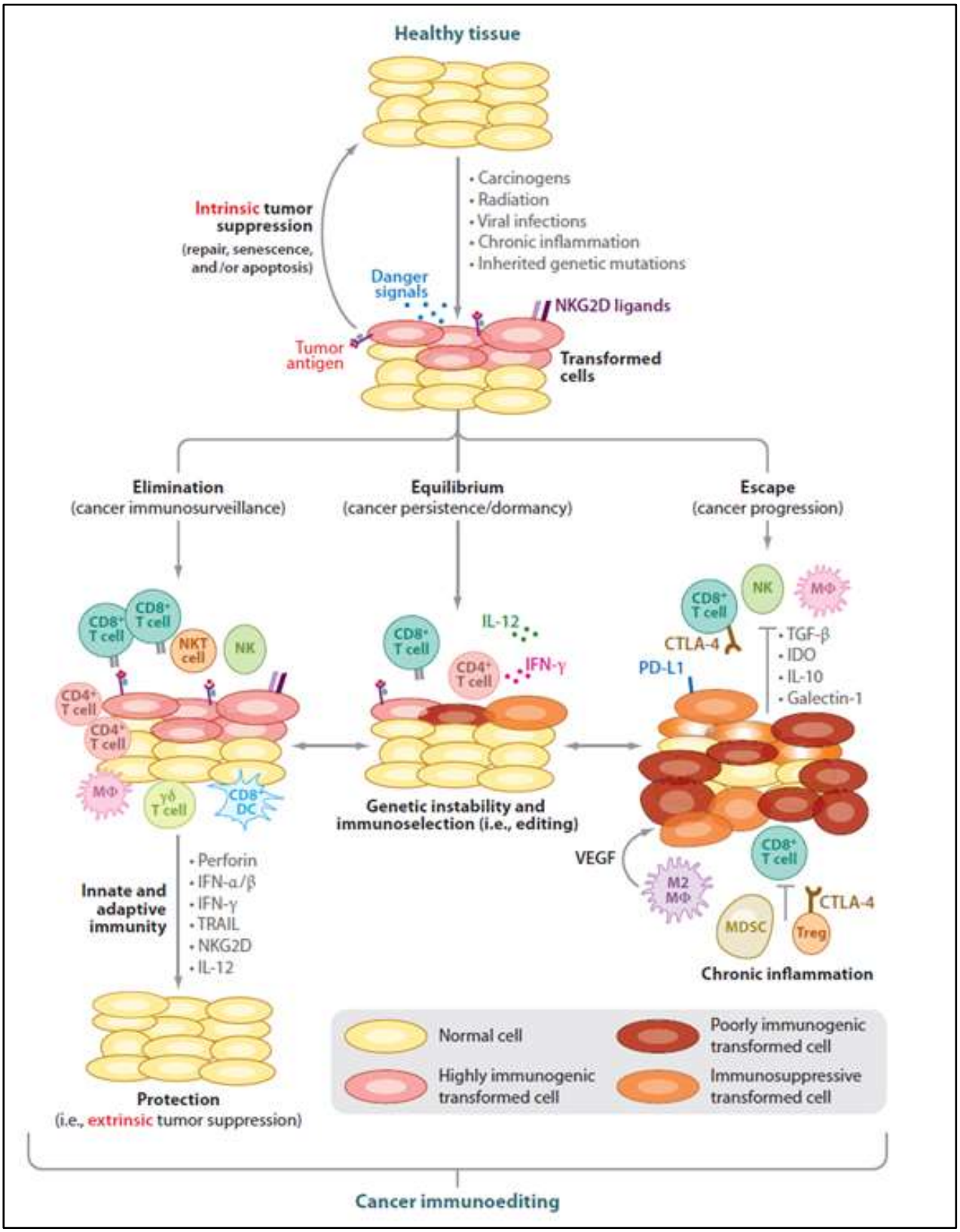

Figure 6: Cancer immunosurveillance and immunoediting. (Adapted from Matthew D Vesely, 2011)

Inflammation

Inflammation is an adaptive immune response which is triggered by noxious stimuli such as infection and tissue injury. At a basic level, infection or tissue injury triggers acute inflammatory response which recruits co-ordinated delivery of plasma and leukocytes. Receptors of innate immune response such as Toll-like receptors (TLRs) and nucleotide-binding oligomerization-domain protein (NOD) like receptors (NLRs) are involved in this kind of response (Barton 2008). Tissue resident macrophages and mast cells recognize the infection and lead to production of various inflammatory cytokines and mediators to elicit an inflammatory response and then the plasma proteins and leukocytes mainly neutrophils which are restricted to the blood vessels gain an access to the extravascular tissues at the infection site (Medzhitov 2008). Neutrophils release toxic contents of their granules such as reactive oxygen species (ROS) and reactive nitrogen species (RNS) cathepsin G, proteinase 3 and elastase (Nathan 2006). The switch in lipid mediators from pro-inflammatory prostaglandins to anti-inflammatory lipoxins is important for converting inflammation to resolution. If the acute inflammatory response fails to destroy pathogen the inflammatory process persists. During infection infiltration of neutrophil is replaced by macrophages and T cells. If macrophages and T cells are not sufficient to confer the effect then a chronic inflammation state ensues which includes formation of granulomas and tertiary lymphoid tissues (Drayton, Liao et al. 2006). Autoimmune response can cause chronic inflammatory condition.

Inflammatory Pathway

There are two important participants in process of inflammation, inducers and mediators. Inducers are the signals that initiate inflammation by activating specialized sensors, hence production of mediators, whereas mediators are responsible for altering the functional states of tissue and organs which are the effectors of inflammation.

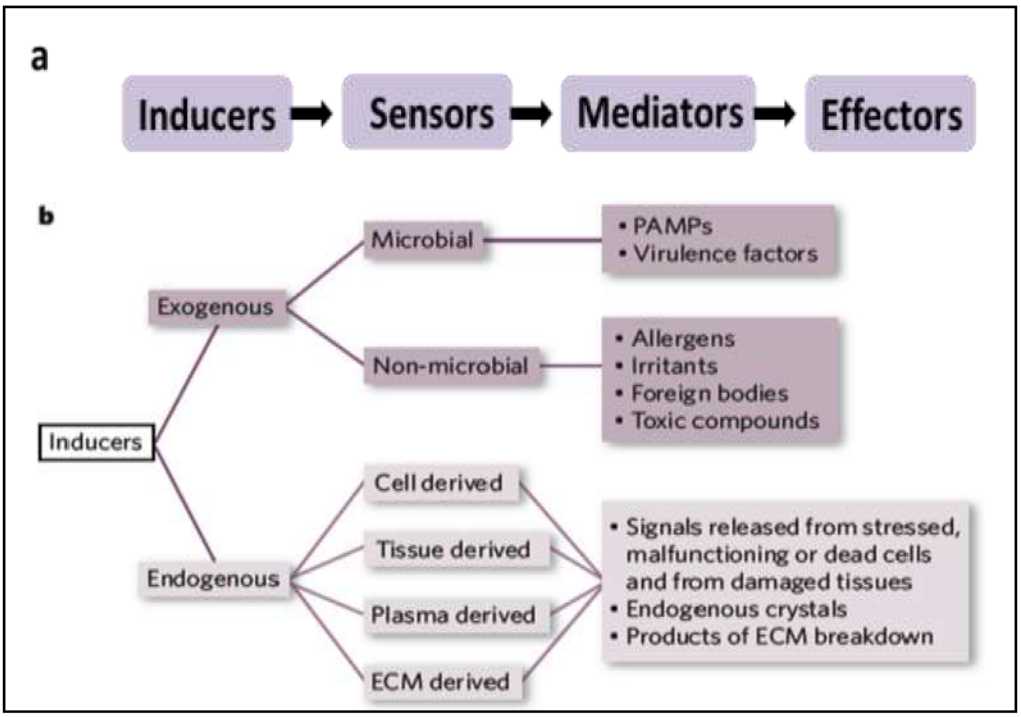

Figure 7: The Inflammatory Pathway. a) Inflammatory pathway consists of inducers, sensors, mediators and effectors. b) Further classifications of the inflammatory pathway. (Adapted from Medzhitov R, Nature, 2008)

Inducers can be exogenous or endogenous. Exogenous inducers are classified in two groups: microbial and non-microbial. There are two classes of microbial inducers: PAMPs and virulence factors. Non-microbial exogenous inducers of inflammation include allergens, irritants, toxic compounds and foreign bodies. Endogenous inducers of inflammation are the signals which are produced by stressed, damaged, malfunctioning tissues. A plasma-derived regulator of inflammation, the Hageman factor (factor XII) activates in contact with collagen and other components of extracellular matrix and upon activation they act as a sensor of vascular damage and initiates the four proteolytic cascades that generate inflammatory mediators: the kallikrein-kinin cascade, the coagulation cascade, the fibrinolytic cascade and the complement cascade (Majno 2004).

Mediators are produced by inducers of inflammation and they are the downstream effectors of inflammatory pathway. These mediators are mainly involved in vasculature and in recruiting leukocytes. They are derived from plasma proteins or they are secreted by cells. Based on their biochemical properties inflammatory mediators can be classified into seven groups: vasoactive amines, vasoactive peptides, fragments of complement components, lipid mediators, cytokines, chemokines and proteolytic enzymes (Majno 2004).

The effectors of inflammatory response are the tissue and cells, the functional states of these effectors are affected by the mediators of inflammatory pathways. Generally an inflammation response is presumably engaged whenever tissue malfunctions are detected. Whatever is the cause of inflammation, its purpose is to sequester the source of irritation, to allow the host to adapt to the unusual environment, and ultimately, to restore the function and homeostasis to the tissue.

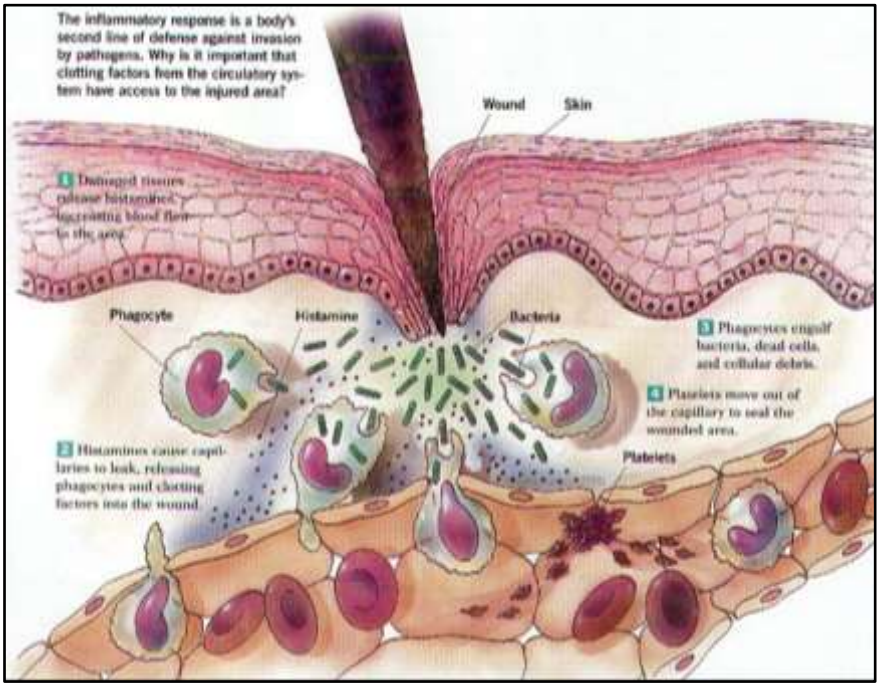

Figure 8: Steps of the inflammatory immune response.

Inflammation and Cancer: Two Faces of Same Coin

The functional relationship between inflammation and cancer is nothing new. In 1863, Virchow hypothesized the origin of cancer was at the sites of chronic inflammation (Balkwill and Mantovani 2001). Inflammation responses orchestrate tumor development at different stages, including initiation, promotion, malignant conversion, invasion, and metastasis. Proliferating cells that sustain DNA damage and mutagenic assault continue to proliferate in inflammatory condition that supports their continuous growth. In other word tumors act as wounds that fail to heal (Dvorak 1986). Epidemiological evidence indicates a reflexive relation between inflammation and a predisposition for the development of cancer, i.e., long-term inflammation can cause dysplasia. Nearly 15% of the world wide cancer cases are associated with microbial infection (Kuper, Adami et al. 2000). In the middle of 19th century Virchow first observed that many tumors for which infection is not the predisposing factor as

in mammary adenocarcinoma, show a lymphophoreticular infiltrate. This type of tumors have activated macrophages and fibroblasts, in addition to a gene expression profile with an inflammatory signature (Rakoff-Nahoum 2006). To prevent familial adenomatous polyposis (FAP), non-steroidal anti-inflammatory drugs (NSAIDs) are used and with the use of these drugs role of inflammation came up (Ulrich, Bigler et al. 2006). Thus cancer and inflammation are linked by epidemiological, histopathological, and inflammatory profiles.

Inflammation Can Cause Cancer

The chronic inflammatory states associated with infection and irritation can result in tumor initiation. During the process of fight against microbial infections reactive oxygen intermediates (ROI), reactive nitrogen intermediates (RNI), are produced which leads to oxidative damage and nitration of DNA bases which in turn increases the risk of DNA mutation (Hussain, Hofseth et al. 2003). Under physiological condition, inflammation mediates tissue repair, but as an extension it may play a contrasting role in providing survival and proliferative signals to the tumor initiated cells, thus promoting tumor progression. The Wnt/β-catenin pathway plays a role in both in maintaining the steady state proliferative compartment as well as in tumorigenesis of tissues (Beachy, Karhadkar et al. 2004). Thus, in the presence of initiation and both tissue injury and massive cell death, inflammatory response activates which leads to tumor promotion.

Cancer Can Cause Inflammation

Coussens and Hanahan have described that tumor growth is biphasic (Coussens, Raymond et al. 1999). In the first phase body treats tumor as wounds. This phase is indicated as physiological tissue repair phase. In the later phase the pro-inflammatory factors like MMPs are under control of tumor themselves (Lin and Karin 2007). A similar transition in the regulation of inflammatory response by early vs late tumors may be at hand in spontaneous intestinal tumorigenesis in both mice and in humans. Playing a critical role in tumor growth, inflammatory response can even have a role in tumor progression by mediating angiogenesis.

Cancer and Inflammation: Friend or Foe?

It is the expression of various immune mediators and modulators and the abundance and activation of different cell types in the tumor microenvironment that decides in which direction the balance is favored and whether tumor-promoting inflammation or antitumor immunity will ensue (Smyth, Dunn et al. 2006). Surgery, chemotherapy and radiation are the cancer treatments which cause local or systemic inflammation triggered by tissue injury and cancer cell death. Surgery leads to an activation of infection-related pathways, whereas radio

and chemotherapy results in cancer cell death mostly through necrosis which is a pro-inflammatory form of cell death (Vakkila and Lotze 2004). In case of more conventional chemotherapy, therapy induced inflammation has been found to stimulate antigen presentation by tumor-infiltrating dendritic cells and results in activation of adaptive anti-tumor immunity by producing several cytokines (Apetoh, Ghiringhelli et al. 2007), (Zhang, Bowerman et al. 2007). Therapy induced anti-tumor immunity is noticed in some of the drugs like doxorubicine, etoposide but not with others (Ghiringhelli, Apetoh et al. 2009). These drugs eliminate infiltrating immune and hematopoietic stem cells, which are essential immune function, thus therapy induced antitumor immunity requires small doses of chemotherapy to be effective to escape immunosuppression.

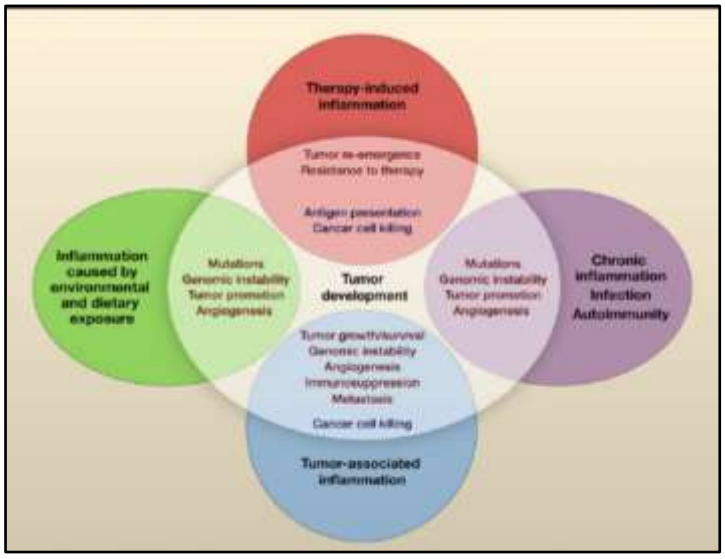

Figure 9: Types of Inflammation in Tumorigenesis and Cancer. (Adapted fromKarin M. et al., Cell, 2010)

Cancer Immunotherapy: Current Paradigm

Since the turn of the century, scientists have tried to understand the interactions between the immune system and cancer cells so that the anti-tumor immunity could be amplified as a mean of cancer therapy. Tumor immunotherapy is emerging by use of several immunomodulatory strategies such as inhibiting immune suppressors or regulatory T cells (Tregs), paralysed APC, suppressive cytokines like TGF-β, and blocking the signalling events which encourage the suppressive phenotype(Gajewski, Meng et al. 2006). Dendritic cells

vaccination can induce immunological as well as clinical responses in cancer patients (Nestle, Banchereau et al. 2001). One of the primary goals of cancer vaccines is to target the immunizing antigens to specific bone marrow derived APCs (Hahn and Weinberg 2002). Recent discoveries show that tumors actively fight back by producing several immunosuppressive factors such as IL-10 (Salazar-Onfray 1999), (Moore, de Waal Malefyt et al. 2001), TGF-β (Gajewski, Meng et al. 2006), (Li, Wan et al. 2006), (Liu, Wong et al. 2007), and VEGF (Gabrilovich, Chen et al. 1996). Agents blocking VEGF, prostaglandins, and estrogen which are the probable contributor of Treg differentiation and/or function in some tumors therefore be therapeutically beneficial through Treg depletion (Curiel 2007). Another promising immunomodulatory approach is to enhance the stimulators of the immune system, like pro-inflammatory cytokines such as IL-2, IFN-γ, IL-12, stimulating the dendritic cells (Liu 1998, Pardoll 1998), which can further drive the immune response towards a specific cytotoxic T cell functioning and activation of NK cells (Rosenberg, Spiess et al. 1986), (Hahn and Weinberg 2002).

Inflammation and cancer, which share several signalling pathways and regulatory mechanisms and the interplay between these two systems, have clearly shown the involvement of inflammatory processes in malignant disease (Vendramini-Costa and Carvalho 2012), (Servais and Erez 2013), (Kundu and Surh 2012), (Sethi, Shanmugam et al. 2012). This is an attractive target for an important immunotherapeutic approach for cancer therapy, suggesting that the inflammatory cells and inflammatory mediators in the tumor microenvironment may be targets for treatment or prevention, and therefore anti-inflammatory drugs may be useful in cancer prevention and treatment (Balkwill and Mantovani 2010). Considering crucial role of inflammatory mediators and the regulators of chronic inflammation in tumor development and in generating an inflammatory tumor microenvironment, anti-inflammatory therapeutics play a promising role in designing efficient therapeutic strategies which can be used in the treatment of malignant diseases and vice versa. Therefore the therapeutics with anti-tumor properties can be used in inflammatory conditions and those with anti-inflammatory properties can be used for the treatment of cancer.

Importance of cyclo-oxygenases and COX-derived prostaglandins in Cancer and in Inflammation

Cyclooxygenases (COXs), also known as prostaglandin-endoperoxide synthase (PTGS) are the enzymes that regulate the biosynthesis of an important family of biological mediators called prostanoids such as prostaglandins, prostacyclin and thrombaxane. They catalyse the first two biochemical reactions in the conversion of arachidonic acid (AA) into prostanoids.

There are several reports suggesting that cyclooxygenases and cyclooxygenase-derived prostaglandins are actively involved in cancer as well as inflammation. A range of human tumors express high levels of cyclooxygenase-2 (COX-2) (Shono, Tofilon et al. 2001), (Wolff, Saukkonen et al. 1998), (Joki, Heese et al. 2000). COX-2 promotes prostaglandin E2 (PGE2) production in the tumor microenvironment and this PGE2 in turn suppresses DC differentiation and function (Sombroek, Stam et al. 2002), (Akasaki, Liu et al. 2004). COX-2 inhibitors are able to suppress human tumor growth in mice (Leahy, Ornberg et al. 2002). Overexpression of the pro-inflammatory mediator COX-2 is a common characteristic of several pre-malignant and malignant cases involving organs like bladder, colon, breasts, lungs, prostate, stomach (Zitvogel, Tesniere et al. 2006). Chemotherapeutic effect in colon cancer has been achieved by inhibiting COX-2. COX-2 results in over production of PGE2 which is thought to have a major role in promoting angiogenesis, through induction of VEGF (Brown and DuBois 2005). Moreover COX-2 and prostanoids especially PGE2, suppress anti-tumor immunity by suppressing macrophage-mediated or T cell-mediated tumor destruction by polarizing the balance of T helper cell responses towards Th2 cell responses. In lung cancer selective inhibition of COX-2 is able to restore the tumor-induced imbalance between IL-10 (Th2 cytokine) and IL-12 (Th1 cytokine) in mice (Stolina, Sharma et al. 2000) and even can restore paralysed mononuclear-cell function in head and neck cancer patients (Lang, Lauffer et al. 2003). In inflammatory setting, the inducible form of cyclooxygenase, i.e., COX-2 is detected in a variety of cells, resulting in high amount production of pro-inflammatory and cytotoxic PGs, playing an important role by enhancing the blood flow to the healing area of injured tissues. Unfortunately release of PGs by inducible isoforms of COX is associated with several diseases. Arachidonic acid is metabolized by cyclooxygenases (COX1 and COX-2) to form eicosanoids which produce prostaglandings and these prostaglandins in turn cause vasodilation. PGE2 is hyperalgesic and a potent inducer of fever (Higgs, Moncada et al. 1984).

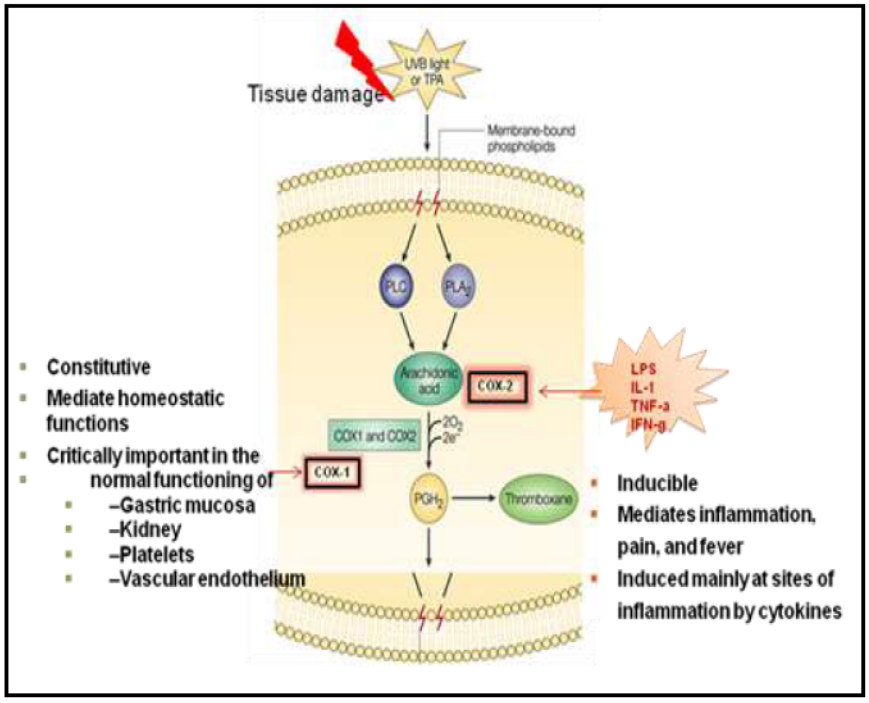

Figure 10: Prostaglandin synthesis from arachidonic acid by cyclo-oxygenases. (Adapted from G. Tim Bowden, Nature Reviews Cancer, 2004)

Cyclooxygenases: Structural and Functional Insights

Cyclooxygenase (COX) also known as prostaglandin H synthase is the key enzyme required for conversion of arachidonic acid (AA) to prostaglandins (PGs). Among three isoforms of this enzyme, cyclooxygenase-1 (COX-1) is constitutively expressed in many tissues and they participate in tissue homeostasis, whereas cyclooxygenase 2 (COX-2) induced by pro-inflammatory cytokines, tumor promoters, oncogenes, and growth factors (Vane, Bakhle et al. 1998), (Dubois, Abramson et al. 1998), (Crofford 1997). Recently identified another isoform COX-3, exhibits the catalytic features of COX-1 and COX-2 (Chandrasekharan, Dai et al. 2002). Human COX-1 and COX-2 are homodimers and they are of 576 and 581 amino acids respectively. Both the enzymes contain three high mannose oligosaccharides, one of which is able to fold proteins. Only COX-2 contains the fourth oligosaccharide which regulates its degradation. There is around 60% homology in the structure of COX-1 and COX-2. Each subunit of the dimer is having three domains, residues 34-72: the epidermal growth factor domain, residues 73-116: the membrane binding domain, and the catalytic domain. This catalytic domain comprises the most of the protein containing the cyclooxygenase and peroxidase function on either side of heme prosthetic group (Smith, DeWitt et al. 2000), (Rouzer and Marnett 2003), (Mbonye, Yuan et al. 2008).

COX-1 is constitutively expressed in resident inflammatory cells, and studies confirm their role in multiple inflammatory models. COX-2 plays a major role in resolution of inflammatory response that is necessary for healing of gastric ulcers (Rouzer and Marnett 2009). Ulceration is observed with a combination of COX-1 and COX-2 inhibitors, suggesting reduction in global PGs is more important than inhibiting a specific COX. Studies with COX-2 knockout mice demonstrate the homeostatic role of this enzyme. Genetic deletion of COX-2 caused a severe dampening of development of postnatal kidney and female knockout mice are no more fertile due to impaired ovulation and embryo implantation (Lipsky, Brooks et al. 2000), (Langenbach, Loftin et al. 1999). It is seen that prolonged use of COX-2 selective inhibitors results in cardiotoxicity which further confirms the homeostatic role of COX-2 (Grosser, Fries et al. 2006), (Marnett 2009). COX-1 and COX-2 are not functionally interchangeable at the protein level. An explanation for the difference in isoform function may be that COX-2 for their activation requires much lower concentrations of hydroperoxide than does COX-1(Kulmacz 2005). Another reason might be COX-2 has wider substrate specificity. Thus, it is clear that the original „COX-2 hypothesis" ascribing a homeostatic function to COX-1 and a patho-physiologic function to COX-2is oversimplified.

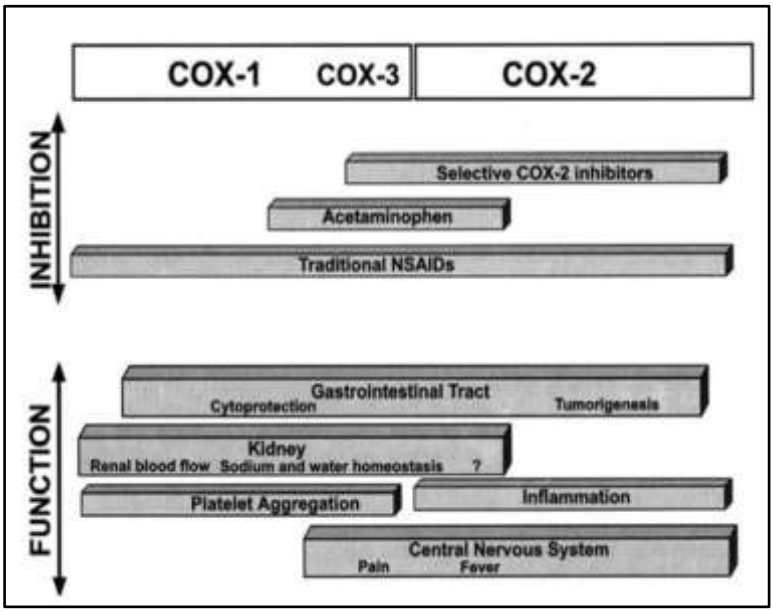

Figure 11: Proposed functions of cyclooxygenase derived PGs. COX-1 participates in physiological hoemostasis, COX-2 is expressed under inflammatory conditions. COX-3 is the splice variant of COX-1. (Adapted from Joan Claria, Current pharmaceutical Design, 2003)

Inhibition of the COX Pathways

COX is the main pharmacological target for nonsteroidal anti-inflammatory drugs (NSAIDs). Vane, Ferreia *et al.* and Smith *et al.* in 1971 first reported abrogation of PGs production by directly inhibiting COX enzymes (Vane 1971), (Ferreira, Moncada et al. 1971). At present worldwide NSAIDs are among the most widely prescribed pharmaceutical drugs to treat pain, fever, inflammation (Thun, Henley et al. 2002). Since COX-1 derived PGs are involved in housekeeping functions and COX-2 derived PGs are in inflammation, NSAID gastrotoxicity to be the consequence of inhibiting both the enzymes by NSAIDs such as aspirin, melofenamate etc. At the site of inflammation (COX-2 activity) the dose required for inhibiting PG biosynthesis also hamper PG production in the gastrointestinal and renal system (COX-1 activity). A new class of compounds which selectively inhibit COX-2 without altering COX-1 dependent PG biosynthesis is developed (Gilroy, Tomlinson et al. 1998), (Smith, Zhang et al. 1998), (Warner, Giuliano et al. 1999). There are *in-vitro* evidence proving that this new generation of anti-inflammatory drugs selectively inhibit COX-2and showed to be as effective as standard NSAIDs in several *in-vivo* inflammatory models (Smith, Zhang et al. 1998),(Warner, Giuliano et al. 1999). There are two selective COX-2 inhibitors (COXIBS) celecoxib and refecoxib are in market for past few years, proven to provide significant relief in osteoarthritis and rheumatoid arthritis, (Simon, Lanza et al. 1998), (Simon, Weaver et al. 1999), (Feldman and McMahon 2000). A second generation of selective COX-2 inhibitors, i.e., valdecoxib and etoricoxib is currently under evaluation in rheumatoid arthritis and osteoarthritis patients.

Targeting COX-2 Expression by Natural Compounds

In autoimmune diseases, it is involved in degenerative functions whereas it correlates with poor prognosis is cancer. Prolonged administration of COX-2 inhibitors has been ineffectual for chemopreventive and chemotherapeutic purposes since the risks prevail over benefits. The classical COX-2 inhibitors may cause severe side effects and efforts are underway to identify alternative chemo preventive approaches. Thus, the current concern is that direct COX-2 enzymatic inhibition might not represent a negligible clinical strategy to target COX-2. COX-2 is a pro-inflammatory immediate early response gene which might encode for cytokines, chemokines and proto-oncogenes.COX-2expression is highly regulated at transcriptional and post-transcriptional levels. As COX-2 expression implies the existence of multiple level of modulation, thus targeting COX-2 expression may represent a promising strategy, by gaining therapeutic benefits while avoiding the severe side effects. Thus, the complex multi-step regulation of COX-2 gene expression offers an alternative to the challenges of COX-2

enzymatic inhibition. This allows the consideration of COX-2 expression as a more versatile target to modulate the wide array of its enzymatic functions, thus potentially contributing new perspectives in therapeutic and chemopreventive strategies. Therefore, the regulatory events which are the determinants of COX-2 expression, in its biological functioning, can be intervened by therapeutic approaches.

Determinants of COX-2 Expression

Transcriptional Regulation

COX-2 promoter contains a number of upstream regulatory sequences specific for binding with a variety of transcription factors, such as NF-κB, the SP-1transcription factor (SP-1), the cAMP responsive element binding protein (CRE), the transcription factor 4 (TCF4), the CCAAT/enhancer-binding protein beta (c/EPB), and the activator protein 1 (AP-1). COX-1 family member lacks characteristic TATA and GC boxes in its promoter region.COX-2 promoter implicates the participation of several kinase-mediated signal transduction mechanisms including mitogen-activated protein kinases (MAPKs), c-Jun NH2-terminal kinase (JNK), p38 and the extracellular signal regulated protein kinases 1/2 (ERK). A wide range of stimuli such as pathogen associated molecular patterns (PAMPs) and pro-inflammatory cytokines may trigger these intracellular signalling pathways, and downstream transcription of COX-2.Activation of COX-2 transcriptional cascade can be altered by modulating the three-dimensional conformation of chromatin due to altered methylation status, or by interfering with the binding of transcription factors, or by altering the expression of regulatory factors required for the transactivation of COX-2. Understanding the transcriptional regulation of COX-2 is important for the designing the therapeutics.

Post-transcriptional Regulation

Post-transcriptional regulation is the process of regulation of the stability of an mRNA. Stability of COX-2 is regulated by two unique mechanisms, first is the presence of 3′-untranslatedregion (3′-UTR) which contains a number of copies of the highly conserved cis-acting consensus motif AAUAAA (AU-rich elements) or AREs and several ARE binding proteins which modulate the physical accessibility and stability of the target mRNAs for translation, second is the regulation by several microRNA (miRNA) such as mir-101a, mir-26b, mir-16 and many others which regulate the stability of COX-2 transcripts, and help to maintain the mRNA turn over and mRNA half-life differentially, depending on the cell type and their inflammatory status. These regulatory mechanisms will maintain the mRNA turnover and silence the COX-2 expression under normal conditions. Thus interfering with

the regulatory events at the post-transcriptional level may serve as potential tool to modulate the expression of COX-2 in various pathological situations.

Post-translational Regulation

There is an interesting role of different post-translational modifications such as N-glycosylation, S-nitrosylation, phosphorylation, and acetylation in maintaining stability and integrity of COX-2 protein. Understanding of the role of these regulatory events is not clear with respect to the emergence of inflammatory and pro-tumoral conditions. Thus better understanding of the post translational steps of COX-2 might implicate potential identification of novel chemopreventive agents acting through the modulation of the post-translational modifications.

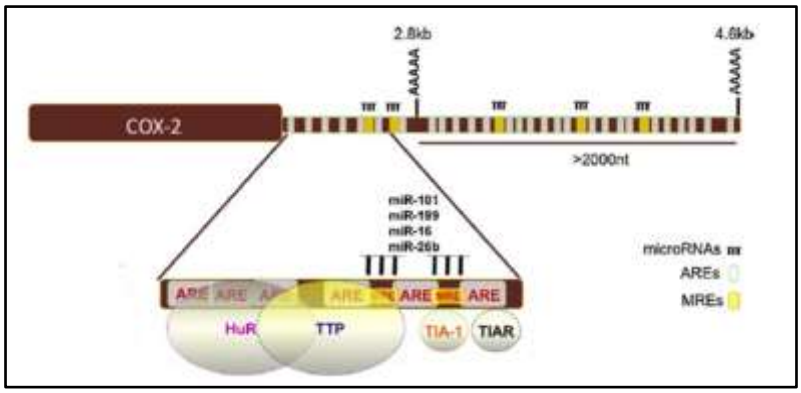

Figure 12: COX-2 Gene Expression. COX-2 transcripts present two alternative sites of polyadenylation, referred as proximal and distal, leading to the formation of transcripts of 2.8 and 4.6 kb, respectively. The long transcript contains an additional sequence of >2000 nt comprising 22 known additional AU-rich elements (AREs). The proximal 116 nt region containing 6 AREs has, however, been characterized as a modulator of mRNA turnover and translation. The trans-acting factors HuR and TTP compete for binding to partially overlapping sequences, thus promoting mRNA stabilization or decay depending on their abundance and level of activity. The two translational silencers TIA-1 and TIAR recognize and bind sequences contained in the same regions, as do some microRNAs. All these factors are believed to form a multimeric protein complex. (Adapted from C. Cerella et al., Biochemical Pharmacology, 2010)

Phytotherapy: A Power of Nature to Cure Immuno-Inflammatory Pathologies and Cancer

The emerging integrative model of cancer treatment recognizes the beneficial role of botanical medicine. A prominent group of effective cancer chemopreventive drugs are available from natural source which exerts low toxicity while processing apparent benefit in the process of disease progression. Herbal terpenoids supress cell proliferation through their anti-neoplastic functions and induces apoptosis. The important molecular targets of chemotherapy are the transcriptional molecules of NF-κB, MEK-ERK and PI3K/Akt/mTOR pathways, which regulate the apoptosis pathways. Recent studies show that induction of pro-apoptototic protein NAG-1 is an important step which decides the anti-carcinogenic property of different herbal compounds (Ko, Leung et al. 2007). In the process of metastasis of cancer the major contributors are angiogenesis, oxidative stress and inflammation, which could be efficiently modulated by several novel plant agents (Ko and Auyeung 2013). The signaling pathways in carcinogenesis could be pre dominantly altered by natural products and novel herbal compounds. In addition of their use in complementary and alternative medicines (CAM), herbal medicines and nutritional supplements are used in combination for symtom management and improving the quality of life. There are several phytosubstances, the flavonoids (genistein, daidzein, quercetin and glycetin), the epigallocatechin-gallate (green tea), the carotenoid lycopene, the polyphenols curcumin, resveratrol which are being used for cancer treatment (Von Low, Perabo et al. 2007). Advances in eliciting the cellular and molecular mechanisms during anti-tumoigenic process of phytotherapeutics will be of important clinical significance to exert the clinical benefit and reduce the adverse effects of drugs in cancer patients and thus improving the quality of life of the cancer patients.

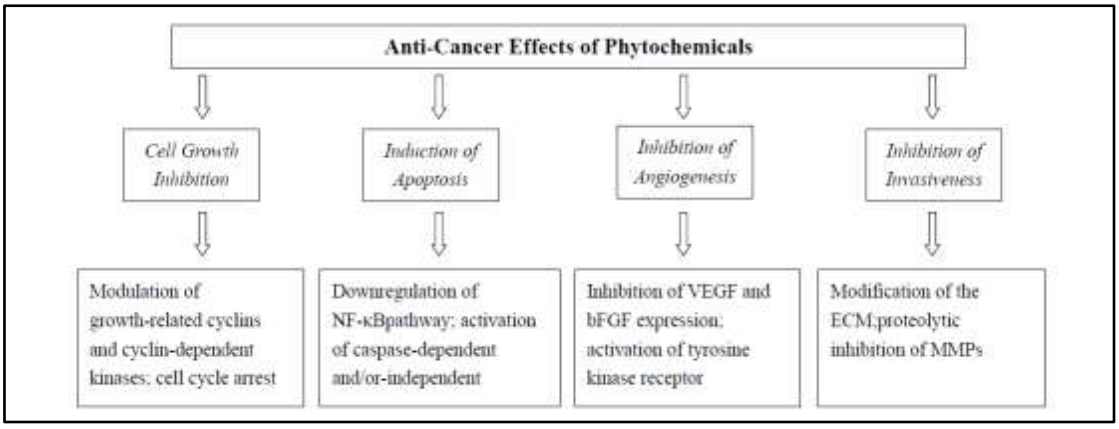

Figure 13: Anti-cancer effects of Phytochemicals. (Adapted from Joshua K Kao, Current Pharmaceutical design, 2013)

Mode of Action	Functional Part	Original
Cytotoxic	Coptisine	*Chelidonium majus*
Apoptotic	Hesperidin	*Citrus spp.*
Anti-proliferation	Water extract	*Coptis chinensis*
Apoptotic	Berberine	*Coptis chinensis*
Anti-inflammatory, apoptotic	Ethanol extract	*Ganoderma lucidum*
Apoptotic	Sulfur-compound	Garlic (*Allium sativum*)
Anti-inflammatory	Berberine	Genera *Berberis* and *Coptis*
Anti-proliferation	Isoliquiritigenin	*Glycyrrhiza uralensis*
Apoptotic	Isoliquiritigenin	*Glycyrrhiza uralensis*
Anti-proliferative, apoptosis	Polyphenols	Green tea (*Camellia sinensis*)
Anti-proliferative	Anthraquinones	*Hemerocallis fulva*
Anti-proliferative, apoptosis	Magnolol	*Magnolia officinalis*
Apoptotic	Thymoquinone	*Nigella sativa*
Anti-proliferative, apoptotic	Chios mastic gum	*Pistacia lentiscus* L. var. *chia*
Anti-proliferative, apoptotic	Oridonin	*Rabdosia rubescens*
Apoptotic	Diosgenin	Seeds of *Trigonella foenum graecum* Linn, *Dioscorea spp.*
Anti-proliferative	Silibinin	*Silybum marianum*
Anti-proliferative, apoptosis	Genistein	Soy (*Glycine max*)
Anti-proliferative, anti-inflammatory	Saponin	Soy (*Glycine max*)

Table.1 List of some medicinal herbal products. (Adapted from Joshua K Kao, Current Pharmaceutical design, 2013)

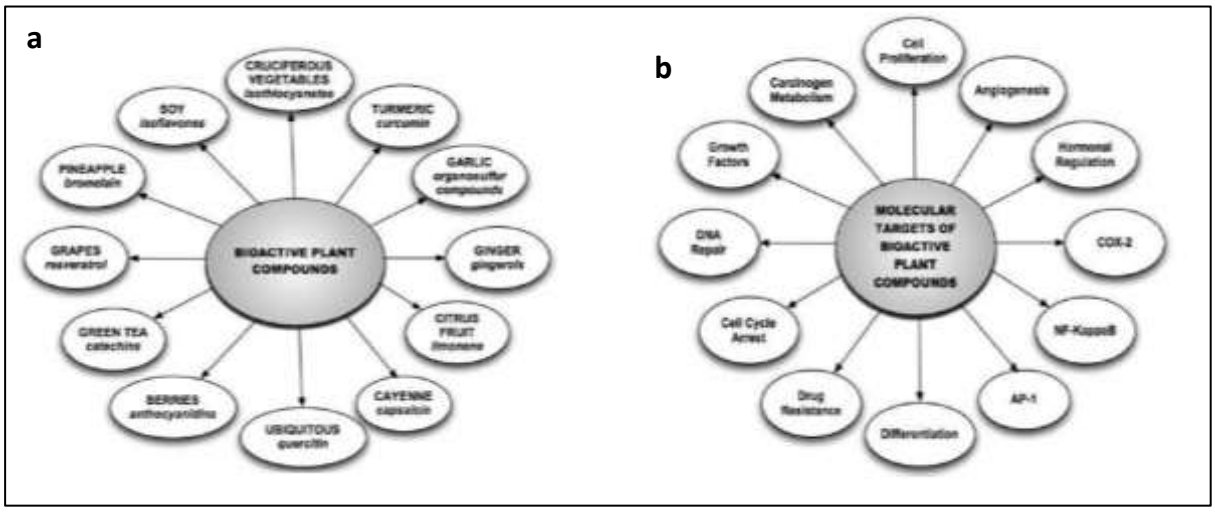

Figure 14: Phytotherapy strategy. a) Bioactive compounds b) Molecular targets of bioactive compounds (Adapted from Treasure et al., Seminars in Oncology Nursing, 2005)

Conventional Oncology and *Viscum album*

Viscum album (*Viscum album L.*) is a traditional phytomedicine of Europe, commonly known as European mistletoe, also found in Western and Southern Asia and it is the most thoroughly studied complementary treatments in Europe. Several systemic reviews and meta-analyses have found that viscum treatment is beneficial for cancer patients in terms of survival, improved quality of life and reduce side effects of conventional anticancer therapies. Several study supports that plant lectins, i.e. carbohydrate-binding proteins, exert remarkable antitumor properties (Liu, Bian et al. 2010), (Liu, Luo et al. 2013), (Zhang, Chen et al. 2012). Schink et al., in a randomised clinical trial, demonstrated that perioperative infusion of Iscador® can inhibit NK cell activity associated with major surgery, which favours haematogenic tumor cell dissemination (Schink, Troger et al. 2007).

Quality of life and *Viscum album*

Quality of life and the safety of adjuvant mistletoe therapy in gastric cancer patients were tested in a randomised clinical trial (Kim, Yook et al. 2012). The additional mistletoe therapy was found to be safe and associated with improved quality of life in these gastric cancer patients. In a study of 70 cancer patients with different digestive tract cancers, use of mistletoe drug- Isorel® showed improved immune competence and an improved overall health

status of cancer patients undergoing conventional anti-tumor therapies (Enesel, Acalovschi et al. 2005), (Galun 2015).

Viscum album

Viscum album has been used in European countries as sole intervention or as complement to the conventional cancer therapies for more than eighty years (Bock, Friedel et al. 2004), (Kroz, Kienle et al. 2014). It is a semiparasitic shrub that grows on other trees. Mistletoelectin belong to the families Loranthaceae and Viscaceae which are both are taxonomically related (Lyu and Park 2006). The pharmacologically applicable components of European mistletoe are lectins, polysaccharides, alkaloids, lipids, triterpenes, peptides, vesicles, flavonoids, visalbcBA (chitin binding agglutinin) (Khwaja, Dias et al. 1986), (Hajto, Hostanska et al. 1989), (Mueller and Anderer 1990), (Park, Hyun et al. 1998). Mistletoe lectin share hetero-dimeric glycoproteins comprising two polypeptide chains: a carbohydrate-binding B-chain which has a capability of binding to cell surface glycol conjugates which permits protein to enter the cell and the catalytic A-chain which acts as ribosome-inactivating substance and hence hinders protein synthesis intracellularly by removing an adenine residue from the 28SrRNA of 60S subunit of the ribosome (Peumans, Verhaert et al. 1996). The extract acts not only as immunostimulatory drug but also have cytotoxic properties and DNA-stabilising properties (Bussing, Regnery et al. 1995). It also stimulates the immune system *in vivo* and *in vitro* by activating monocytes, macrophages, T cells, dendritic cells, granulocytes, NK cells further induces several cytokines like IL-1, IL-2, IL-4, IL-5, IL-6, IL-8, IL10, IL-12, GMCSF, IFN-γ and TNF-α (Thies, Nugel et al. 2005).

Mythological Aspect

The European mistletoe is a pharmaceutical plant and a symbol in mythology. Depending on host specificity, there are three host races existing in Europe, they are 1. V. Album album-grows on a wide variety of deciduous trees. 2. V. a. abietis (fir). 3. V. a. austriacum (Pine). European mistletoe, i.e., is the first plant which was termed as “mistletoe”. Theopharastos (371-286 BC) recognised that mistletoe is spread to the trees by birds and never touched the earth. During the dead winters in Europe when branches of Oak trees were bare, mistletoe is still green even considering the fact of not having roots on earth, made the Celtic Druids believe mistletoe as sacred as ever-lasting life. According to G. P. Secundus (23-79 AC) this plant was considered to be an antidote for poisons and the plant became a miracle because of its ability to cure each illness. In ancient times this mythical plant was used in combination

with aromatic compounds and the surmise was the plant served protection against bad dreams, lighting etc (Büssing 2003).

Mistletoe as a Remedy

Diosorides, the Greek author and physician (15-85 AC) reported that during 460-377 BC spleen related diseases were treated using Oak tree mistletoe. During 23-79 AC, Plinius explained the beneficial role of mistletoe in the treatment of infertility, ulcers, epilepsy. Platonist around 150 AC described the utilization of mistletoe to treat tumors. During 1731 mistletoe was used for various treatments including labour pain, guts and deworming children. Mistletoe treatment seemed to be beneficial for mumps, leprosy, and hepatitis. During 18th Century this remedy was used for Oedema and heart weakness. In spite of having a strong historical background of mistletoe, in the 19th Century scientific community rejected mistletoe remedy. However, the interest was re awakened in the 20th century when Ganltier (1907, 1910) demonstrated oral/subcutaneous administration of fresh mistletoe (L. Extract) to cure blood pressure related issues both in animal and human. In 1920, the founder of anthroposophy, Rudolf Steiner, introduced L. as an anti- cancer remedy (Büssing 2003).

Preparation of therapeutic preparation of *Viscum album*

Iscador® is the commercial preparation of VA preparations. It is prepared as an aqueous extract of the whole mistletoe plant with a formulated fermentation with the bacterium *Lactobacillus plantarum*. The product is then mixed and filtered to remove the bacteria before being standardized and packaged in ampules for injection. Fermentation of the mistletoe extract alters its medicinal activity to a significant degree and this change is thought to be related to the degradation of the most toxic lectins. It is believed that the efficacy of mistletoe extracts like Iscador are due to a synergy between both its components that are medicinally active when isolated, and those components like polysaccharides that are medicinally inactive, but can interact with the more active constituents to form complexes.

Chemical Compounds in *Viscum album*

Viscotoxins

Viscotoxin shows the structural characteristics of plant α- and β-thionins, which are the cysteine rich residues and highly basic in nature. Seven different isoforms have been identified so far, they are viscotoxin A1, A2, A3, B, B2, C1 and 1-PS. These isoforms are consist of 46 amino acids. , among which 32 have identical amino acids and all isoforms contain 3 disulphide bridges at highly conserved positions which are Cys3/Cys40,

Cys3/Cys32 and Cys16/Cys26). They are compact in structure and highly stable under several denaturing conditions. The phosphate binding site of viscotoxins interferes with cell membrane and dampens their integrity, which is the main reason of their cytotoxic effects, primarily necrosis and later in apoptosis (Schaller, Urech et al. 1998).

Lectins

Three different mistletoe lectins (ML) were identified in *Viscum album* with differential sugar-binding specificities, they are galactose-specific MLI: 115 kDa, diner, galactose- and N-acetyl-D-galactosamine-specific MLII: 60 kDa and *N*-acetyl-D-galactosamine-specific MLIII: 60 kDa. They belong to type 2 ribosome inactivating proteins that consist of a lectin subunit B and a toxophoricA chain, which is RNA *N*-glycosidase. In eukaryotic cells, MLs block protein synthesis by hydrolysing 28S rRNA. They induce apoptotic cell death (Krauspenhaar, Eschenburg et al. 1999), (Wacker, Stoeva et al. 2004).

Polysaccharides

Mistletoe stems and leaves show differences due to the structural difference in the polysaccharides. From these parts of the plant, a highly methylated galacturonan, a pectin (42 kD) and arabinogalactan (110 kD) were isolated. The high-molecular weight arabinogalactan of viscum stimulates $CD4^+$ Th cells proliferation (Stein, Edlund et al. 1999). Stimulation of NK cells by Iscador® is due to its rhamnogalacturonan (Mueller and Anderer 1990).

Liposoluble compounds

Viscum album is rich in triterpenes, includingoleanolic acid, β-amyrinacetate, β-amyrin, the lupanesleupeol, lupeol acetate, betulinic acid and urosolic acid (Jager, Trojan et al. 2009), (Urech 2015).

Flavonoids, Phenylpropanoids and phenolic Acids

Viscum album contains phenolic compounds such as flavonoids, phenolic acids, phenylpropanoids. Evidence of their pharmacological roles in pathologies is of great interest. Lectin-induced apoptosis in cancer cells through increased oxidative stress is due to certain flavonoids. They exert cytotoxic, anti-angiogenic, detoxicative, anti-inflammatory and anti-hormonal effects on cancer cells (Schramm 2015), (Urech 2015).

Class of chemical compounds		Compounds
Proteins	Thionins	Viscotoxins A1, A2, A3, B, B2, C1, 1-PS
	Lectins	Mistletoe lectin I (MLI), II (MLII), III (MLIII) Chitin-binding mistletoe lectin 1 (cbML1), 2 (cbML2), 3 (cbML3)
Peptides	Oligopeptides	Glutathione
Amino acids		Arginine, cystein, γ-aminobutyric acid
Amines		Acetylcholine, choline, tyramine, histamine
Proteoglycans		Arabinogalactan-proteins
Polysaccharides		Methylated poly-1→α4 galacturonic acid, arabinogalactan, rhamnogalacturonan
Fatty acids		Oleic acid, palmitic acid, linoleic acid, linolenic acid, arachidic acid, cerotic acid, stearic acid, lignoceric acid
Phenolic compounds	Flavonoids	2′-hydroxy-4′,6′-dimethoxychaIcone-glucosid, 2′-hydroxy-4′,6′-trimethoxychaIcone-glucosid, 2′-hydroxy-4′,6′ dimethoxy- chalcone-4-O-[apiosyl(1→2)] glucoside, (2R)-5,7-dimethoxyflavanone-4′-O-gIucosid, (2S)-3′,5,7-trimethoxy-flavanone-4′-O-gIucoside, homoeriodictyol-7-Oglucoside, rhamnazin-3,4′-di-O-glucoside, 5,7-dimethoxy-4′-hydroxyflavon *After acid hydrolysis*: homoeriodyctiol, sakuranetin, rhamnazin, isorhamnetin, quercetin, 6 different quercetin methylesters, kaemferol, naringenin
	Phenylpropanoids	Syringenin-4′-O-glucoside (syringin), syringenin-4′-O-apiosyl-1 (Pfeil) 2 glucoside (syringoid), syringaresinol-4,4′-O-glucoside, eleutheroside E, syringaresinol- mono-O-glucoside, sinapic acid, cinnamic acid, rosmarinic acid, caffeic acid, ferulic acid, chlorogenic acid, isochlorogenic acid, syringic acid, p- and m-coumaric acid
	Other phenolic acids	Gallic acid (3,4,5-trihydroxybenzoic acid), digallic acid, para-OH benzoic acid, syringic acid (methylated trihydroxybenzoic acid), salicylic acid, protocatechuic acid (3,4-dihydroxybenzoic acid), vanillic acid (methylated dihydroxybenzoic acid), gentisic acid (2,5 dihydroxybenzoic acid), salicylic acid (2 hydroxybenzoic acid), ellagic acid
Terpenoids	Triterpenoids	Oleanolic acid, betulinic acid, ursolic acid, β-amyrin, β-amyrin acetate, lupeol, lupeol acetate
	Tetraterpenoids	Carotin
Phytosterols	β-sitosterol, stigmasterol	
Inorganic substances	Manganese, potassium, calcium (calcium oxalate)	
Various compounds	Ascorbic acid 7-iso-jasmonic acid, and its precursor 12-oxophytodienoic acid	

Table 2. Chemical compounds identified in the European *Viscum album* L. (Adapted from Konrad Urech, Transl Res Biomed, 2015)

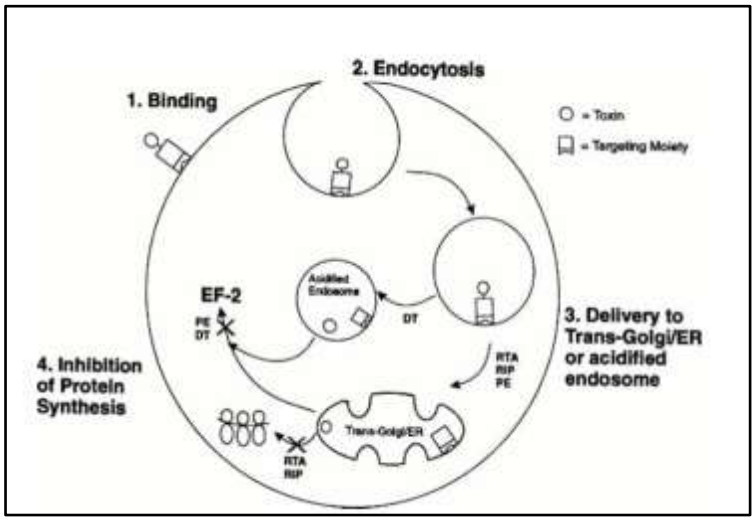

Figure 15: Mechanism of action of type II lectins.

Multifarious Properties of *Viscum album*

Cytotoxicity

Mistletoe lectin can induce apoptosis depending on the apoptosis-associated factor-1 (Apaf-1) pathway by mitochondrial membrane potential (MMP) breakdown and stimulating caspase-3 (Lyu, Choi et al. 2002), (Liu, Luo et al. 2013). JNK can be stimulated by ML-I and leads to translocation of the pro-apoptotic proteins Bax and Bad. Mistletoes induce apoptosis in human peripheral blood lymphocytes, murine lymphocytes, mononuclear leukemia cells MOLT4 and human monocytic THP1 cells (Janssen, Scheffler et al. 1993), (Mockel, Schwarz et al. 1997), (Kim, So et al. 2000). ML-I downregulates Bcl-2 and upregulates TNF-α and hence provoke apoptosis. We have demonstrated that VA Qu Frf, induces significant cell toxicity *in vitro* in the human T cell lines CEM and in monocytic cell lines HL-60 and MM-6 (Duong Van Huyen, Sooryanarayana et al. 2001).

Anti-inflammatory

Diarylheptanoids and flavonoids of mistletoe inhibit LPS-stimulated production of pro-inflammatory cytokines such as TNF-α, IL-6, IL-12p40 in bone marrow-derived dendritic cells (Nhiem, Kiem et al. 2013). Our group have found that VA Qu Spez impedes cytokine-induced PGE2, by selectively inhibiting COX-2 which is transcriptionally activated in response to various pro-inflammatory cytokines (Hegde, Maddur et al. 2011). Further, we have dissected the molecular events of COX-2 regulation recently we observed significant reduction of COX-2 mRNA half-life without influencing its protein stability, clearly suggesting that it induces destabilisation of COX-2 mRNA (Saha, Hegde et al. 2015).

Anti-angiogenic:

Tumor growth can be impedimented by inhibiting angiogenesis. Treating B16L6 melanoma cells with viscum suppressed tumor growth and resulted in DNA fragmentation, nuclear morphological changes, suggesting that it inhibits tumor growth and metastasis by elevating apoptosis and blocking angiogenesis (Park, Lyu et al. 2001). Our group has shown that VA QU Frf induces apoptosis of endothelial cells in human umbilical vein endothelial cells (HUVEC) and in immortalised human venous endothelial cell line (IVEC). In an *in vivo* system it was clearly demonstrated that VA Qu Spez dramatically impedes the vessel score in mice, diminishing angiogenic growth (Elluru, Duong Van Huyen et al. 2009).

Immunomodulatory

Viscum album, significantly enhanced IFN-γ; strongly supports the fact that is an immune modulator (Lyu and Park 2007). Our group has reported that in a murine melanoma model, QU Frf mistletoe preparation significantly inhibited tumor growth and up-regulated IL-12 secretion, which was confirmed by abrogation of IL-12 expression in IL-12 knockout mice (Duong Van Huyen, Delignat et al. 2006). Qu Spez amplifies the expression of several antigen presenting and co-stimulatory molecules on human dendritic cells and additionally induces secretion of pro-inflammatory cytokines such as IL-6 and IL-8 and stimulates proliferation of $CD4^+$ Tcells (Elluru, Duong van Huyen et al. 2008).

***Viscum album*: Clinical Evidence**

Rudolf Steiner, the father of anthroposophy, in 1961 had first propounded the use of mistletoe extracts for cancer treatment. Anthrosopical medicine professes that mistletoe is effective for cancer patients as it delays disease progression, protracts survival time, modulates immune function and overall improves general well-being. Only three among several preparations of mistletoe is tested clinically, they are Iscador®, Eurixor®, and Helixor® (Kleijnen and Knipschild 1994). There are several reports on the use of mistletoe in clinics and their efficacy. However, the proof of results is controversial because of the loopholes in the methodology used to evaluate the effectiveness of complementary medicine.

H.S.LIN (2004) group had taken an attempt to gather information on the efficacy, safety and side effects of standardized mistletoe extract by following “Good Clinical Practice”. This study enrolled 233 patients with 71 ovarian, 68 breast and 94 non-small lung cancer. Among these 233 cancer patients 224 patients were included in the final analysis out of which 115 patients were treated with mistletoe. All patients underwent the conventional chemotherapies.

According to the “Intension to treat principle” analysis, complementarily mistletoe treated patient group had shown a significant improve in the quality of life (QOL) compared to the control group. Concerning the side effects such as fatigue, nausea, anorexia and insomnia, the adverse effects (AES) recorded were fewer than the control group. Most of these effects were self-limiting and harmless, proving the safety of mistletoe use (Piao, Wang et al. 2004).

In 2004, another clinical study reported by U. Mengs, recruiting 272 patients, showed PS76A2, an aqueous mistletoe extract at a dose of 15 ng ML/0.5ml/twice weekly is safe for treatment and improved QOL effectively in breast cancer patients who were receiving adjuvant cyclophosphamide-methotrexate-fluorouracil (CMF). An increase of T helper lymphocytes ($CD4^+$) as well as the ratio between $CD4^+/CD8^+$supported the data on QOL (Semiglasov, Stepula et al. 2004).

Schiernoz team (2008) investigated the safety and efficacy of standardised mistletoe extract HELIXOR® complimentary treatment of breast cancer patients. The study was carried out during a defined after care period of 5 years of the patients through a comparative epidemiological cohort study by random selection of 53 hospitals in Germany including 681 patients. Firstly data showed 56.3% study group versus 70% control group complaints/therapy-related symptoms and secondly disease-related signs was lessened with mistletoe treated patients harmonized to a significantly improved QOL. The adverse drug reactions were self-limiting (Beuth, Schneider et al. 2008).

Physicians often noticed that mistletoe intake have favourable impact on cancer-related fatigue (CRF). In one of the clinical study, a 36-year old Swedish woman with 10-year history of reappearance of breast cancer was suffering from CRF and when the lady was treated with mistletoe beside the conventional therapy, she improved her QOL by reducing fatigue level (Wode, Schneider et al. 2009).

Preclinical and clinical studies exploring the effect of extract recommended strongest beneficial role of VAE in terms of QOL and tolerance to the conventional anti-cancer treatments in gynaecological and breast cancer. VAE was able to elevate survival rate and abrogate tumor especially in mice. There was a strong cytotoxic effects observed on cancer cells in vitro with VAE treatment (Kienle, Glockmann et al. 2009).

A study investigated Post relapse (12 month) disease-free survival rate in Osteosarcoma patients who has a high chance of second relapsing receiving either Etoposide or *Viscum album*. Twenty patients were matriculated in the study. In Etoposide group the median

PRDFS was 4 months, whereas it was prolonged to 39 months in viscum group. Thereby, viscum therapy remains superior to Etoposide treatment (Longhi, Reif et al. 2014).

Mansky et al (2003) conducted a phase I, 2 stage dose-escalation study (Case Study: 02-074) with the intension to test European mistletoe (Eurixor^{TM}) used with an approved antimetabolite chemotherapeutic agent gemcitabine in solid tumor patients. Combination of 1380 mg/m^2 gemitabine and 250 mg mistletoe was the maximum tolerated dose. Out of 44 patients, 33 patients completed minimum 3 cycles of therapy. 6% (n=2) showed partial response, 42% (n=14) developed stable disease, 43% (n=14) improved upon treatment. 37% patients developed non neutropenic fever, whereas control group was associated with 41% fever. Mistletoe/gemcitabine cocktail and gemcitabine alone showed similar hematologic toxicity profile and febrile reaction. There was an ascending trend of ANC with mistletoe treatment (Mansky, Grem et al. 2003), (Mansky, Wallerstedt et al. 2013).

European mistletoe extracts (*L.)* are the most commonly prescribed cancer treatments in Germany *per se* in 2010 (Kroz, Kienle et al. 2014). *In–vitro* and *in-vivo* studies have identified their immunomodulatory and cytostatic effects (Lyu and Park 2006). Today the therapeutic goal is to improve health related QOL and that is acknowledged as an end point in clinical trials. Pharmacological actions of mistletoe lectin are well documented, however, clinical trials evidence was rare and the existing proofs have been criticised. Wide variety of commercial availability of mistletoe impedes the comparative assessment of the benefits of use of the extract in cancer therapy. Difference in the extraction process and manufacturing method, result in the variation of pharmacological or clinical effects of mistletoe (Kleijnen and Knipschild 1994). Consequently complementary treatment with standardised mistletoe extract in cancer can be regarded as safe.

OBJECTIVES

In view of the critical link between inflammation and cancer which share several signalling events, regulatory mechanisms, it is necessary to unravel the molecular and cellular mechanisms of underlying anti-inflammatory and immunomodulatory effect of *Viscum album*, which can provide a better understanding of its immunotherapeutic strategies to develop integrative medicinal approaches to inflammatory pathologies and cancer. Therefore my study addresses the anti-inflammatory and immunomodulatory properties of viscum and the mode of action which in turn can strengthen the beneficial application of viscum in complementary therapy to improve the survival and quality of life of cancer patients. Following are the objectives of my study.

Objective 1: Molecular dissection of *Viscum album* mediated COX-2 inhibition and better understanding of its anti-inflammatory effect.

Objective 2: Exploring the immunomodulatory effects of *Viscum album* by studying differential effect of various preparations of *Viscum album* on maturation and activation of human dendritic cells and T cell response.

Objective 3: Exploring the anti-tumor response of *Viscum album* by understanding their effect on the full spectrum of macrophage polarization.

RESULTS

PLOS ONE

RESEARCH ARTICLE

Viscum album-Mediated COX-2 Inhibition Implicates Destabilization of COX-2 mRNA

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Abstract

Extensive use of Viscum album (VA) preparations in the complementary therapy of cancer and in several other human pathologies has led to an increasing number of cellular and molecular approaches to explore the mechanisms of action of VA. We have recently demonstrated that, VA preparations exert a potent anti-inflammatory effect by selectively down-regulating the COX-2-mediated cytokine-induced secretion of prostaglandin E2 (PGE2), one of the important molecular signatures of inflammatory reactions. In this study, we observed a significant down-regulation of COX-2 protein expression in VA-treated A549 cells however COX-2 mRNA levels were unaltered. Therefore, we hypothesized that VA induces destabilisation of COX-2 mRNA, thereby depleting the available functional COX-2 mRNA for the protein synthesis and for the subsequent secretion of PGE2. To address this question, we analyzed the molecular degradation of COX-2 protein and its corresponding mRNA in A549 cell line. Using cyclohexamide pulse chase experiment, we demonstrate that, COX-2 protein degradation is not affected by the treatment with VA whereas experiments on transcriptional blockade with actinomycin D, revealed a marked reduction in the half life of COX-2 mRNA due to its rapid degradation in the cells treated with VA compared to that in IL-1β-stimulated cells. These results thus demonstrate that VA-mediated inhibition of PGE2 implicates destabilization of COX-2 mRNA.

Introduction

Cyclo-oxygenase-2 (COX-2) is an early response protein, up-regulated during many pathological conditions and human malignancies. It is over expressed in most of the cells upon stimulation with diverse pro-inflammatory stimuli such as pro-inflammatory cytokines, chemokines, infectious agents, bacterial lipopolysaccharide etc. COX-2 is a critical enzyme required for the biosynthesis of prostaglandin E2, one of the important molecular mediators of inflammation [1]. Two other COX isoenzymes, COX-1 and COX-3, catalyze the same kind of reaction. COX-

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1 is an important cyclo-oxygenase family member, and is constitutively expressed in cells and tissues, while precise functions are not known for COX-3, which is expressed only in some specific compartments including brain and spinal cord [2, 3]. The pattern of expression of COX-1 versus COX-2 further regulates their differential functions. COX-1 is constitutively and stably expressed at low levels in many tissues. This ensures a constant production of prostaglandins, which are essentially required for the maintenance of important physiological functions, such as platelet aggregation, normal renal functions and gastric mucosal protection. In contrast, COX-2 is mostly quiescent but the expression can be induced in response to diverse pro-inflammatory and pathogenic stimuli. When stimulated, its expression is high and transient which leads to a burst of prostaglandin production in a regulated time-limited manner [4]. Thus, depending on the COX isoform, the production of the same precursor PGH2 from arachidonic acid differs with respect to the amount and timing of production. This can be differentially decoded by the cells, thereby leading to the activation of various intracellular pathways involving specific classes of prostaglandins and therefore, different responses [5].

Since COX-2 expression is up-regulated during several pathological conditions and human malignancies, strategies controlling the expression and activity of COX-2 have been developed as potent anti-tumor and anti-inflammatory treatments [6–10]. In line with the therapeutic benefit of non steroid anti-inflammatory drugs (NSAID), which are synthetically designed mainly to inhibit the enzymatic activity of COX-2, a diverse spectrum of therapeutics of natural origin such as phytotherapeutics have been characterized to evaluate their potential to inhibit the COX-2 functioning thereby down-regulating the pathological level of prostaglandins. Due to the structural homology between COX-1 and COX-2, most of the NSAID inhibit both the enzymes and thus resulting in several severe side effects due to the inhibition of physiological prostaglandins. Therefore, selective inhibitors of COX-2 are of great interest. Although, a promising class of synthetic COX-2 selective inhibitors called COXIBS have been developed, their therapeutic efficacy is compromised due to various side effects [11, 12]. Interestingly, several phytotherapeutics have been shown to exert therapeutic benefit via selective inhibition of COX-2. These natural molecules have been shown to interfere with the expression and regulatory mechanisms of COX-2 to inhibit its functioning [13, 14].

Viscum album (VA) preparations commonly called as mistletoe extracts, are extensively used as complementary therapeutics in cancer and also in the treatment of several inflammatory pathologies [15–19]. Despite their therapeutic application for several years, the underlying mechanisms are not yet clearly understood. Several lines of evidence have revealed that these preparations exert anti-tumor activities, which involve the cytotoxic properties, induction of apoptosis, inhibition of angiogenesis and several other immunomodulatory and anti-inflammatory mechanisms [20–30]. These properties collectively define the mechanistic basis for the therapeutic benefit of VA preparations. Recently we have shown that, VA preparations exert a potent anti-inflammatory effect by selectively down-regulating the COX-2-mediated cytokine-induced secretion of prostaglandin E2 (PGE2), one of the important molecular signatures of inflammatory reactions [31]. However, the molecular mechanisms associated with the Viscum-mediated COX-2 inhibition are not clear. VA preparations are shown to inhibit the COX-2 protein expression without modulating its expression at mRNA level suggesting a possible effect of VA on post-transcriptional events of COX-2 regulation. Several molecules and phytotherapeutics are known to interfere with the post-transcriptional and post-translation regulation of COX-2 in order to inhibit the COX-2 expression and subsequent reduction of PGE2 [32–34]. Therefore in the current study, we investigated the post-transcriptional and post-translational regulation of COX-2 by analyzing the stability of COX-2 protein and mRNA, which can explain in part, the molecular mechanisms of Viscum-mediated COX-2 inhibition.

Materials and Methods

Viscum album preparations

VA Qu Spez was a kind gift from Weleda AG (Arlesheim, Switzerland). VA Qu Spez is a therapeutic preparation of Viscum album that grows on oak trees and is obtained as an isotonic solution of 10mg/ml formulated in 0.9% NaCl. It is free from endotoxins and contains the standardized levels of mistletoe lectins.

Culture of A549 cells

Human lung adenocarcinoma cell line A549 was a kind gift from Dr. Maria Castedo-Delrieu, Institute Gustave Roussy, Villejuif, France. A549 cells were grown in 75 cm^2 culture flasks in Dulbecco's modified Eagle's medium (DMEM) F-12 (GIBCO, Life Technologies, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FCS) and 50 U/ml penicillin and 50 μg/ml of streptomycin (GIBCO). Cells are incubated at 37°C with 5% CO_2 in humidified atmosphere to obtain the cells of about 80–90% confluence and used for all experiments.

Co- and post- treatment of VA Qu Spez and induction of COX-2

Cells grown in complete medium (DMEM with 10% FCS) were harvested by trypsinisation using 0.5% trypsin (Biological Industries, Kibbutz Beit Haemek, Israel) and were seeded in 12-well culture plates (0.5×10^6/ml cells per well). Wells containing the adherent A549 were then replenished with the complete medium containing recombinant human IL-1β (10 ng/ml) (Immuno Tools, Friesoythe, Germany). In one set of experiment VA Qu Spez is added at the time of addition of IL-1 β (co-treatment) and in another set, we add VA Qu Spez 14 hours after adding IL-1β (post-treatment) and both the sets were incubated until 18 hours at 37° C and 5% CO_2. After 18 hours of incubation cells were harvested by trypsinization and used for the analysis of COX-1/COX-2 protein by flow cytometry.

Analysis of the degradation profile of COX-2 protein by cyclohexamide pulse chase experiment

A549 cells with an appropriate confluency were treated with IL-1β for 18 hours in the presence or absence of VA Qu Spez. To block the protein synthesis 10 μg/ml of cyclohexamide (Sigma-Aldrich, Lyon, France) was added after 90 minutes of addition of IL-1β and then cells were harvested at different time intervals as indicated to achieve a clear pattern of COX-2 degradation. At each time point, expression of remaining COX-2 protein was analyzed by intracellular labelling, by flow cytometry and further validated by western blotting.

Analysis of COX-2 mRNA half-life by actinomycin D pulse chase experiment

A549 cells with an appropriate confluency were treated with IL-1β for 4 hours in the presence or absence of VA Qu Spez. After 4 hours, 10 μg/ml of actinomycin D (Sigma-Aldrich) was added to the cells and cells were harvested by trypsinisation at different time intervals as indicated. Expression of remaining COX-2 mRNA was analyzed by RT-PCR.

Statistical analysis

Densitometric analysis of the immunoblots was performed using BIO-1D analysis software. Values are expressed as arbitrary units. All the observations are expressed as Mean ±SEM and

analyzed using two-way ANOVA. Graph-Pad Prism 5.0 is used for all the statistical analysis. P values less than 0.05 were considered to be statistically significant.

Results

Co-treatment of A549 cell with IL-1β and Viscum album inhibits the cytokine-induced COX-2 expression

Following our observation of the inhibition of cytokine-induced COX-2 expression, we investigated the appropriate window of efficient inhibition by VA. Human lung adenocarcinoma (A549) cells were stimulated with IL-1β for 18 hours in the presence or absence of VA Qu Spez. VA was added to the cells either along with the cytokine (co-treatment) or after 14 hours of IL-1β induction. Flow cytometric analysis of intracellular COX-2 expression demonstrated that VA significantly inhibits cytokine-induced COX-2 expression as measured by mean fluorescent intensity (MFI) only when it is added as a co-treatment with IL-1β but not when it was added after 14 hours (Fig. 1A and 1B). This suggests that, VA-mediated COX-2 inhibition occurs at the early phases of inflammatory process and opens other exploratory avenues to understand the regulatory mechanisms of COX-2 inhibition mediated by VA at the early phase of inflammation.

Inhibition of COX-2 protein expression by Viscum album is independent of modulation of stability of COX-2 protein

In order to address the effect of VA on the molecular stability of COX-2, which could be a potential contributing factor for the observed reduction in COX-2 protein expression, we analyzed the stability of COX-2 protein. A549 cells were stimulated with a pro-inflammatory cytokine IL-1β in the presence and absence of VA Qu Spez. At 18 hours, we observed a significant reduction in COX-2 protein level treated with VA Qu Spez. Further, cells were harvested at different time intervals after blocking the protein synthesis by treating the cells with cyclohexamide and analyzed for COX-2. Flow cytometric analysis of COX-2 protein has revealed that, there is no significant difference in the protein degradation profile of COX-2 in VA-treated and untreated cells after 90 minutes of blocking the protein synthesis (Fig. 2A and Fig. 2B). Further, western blot analysis of COX-2 protein expression at different time intervals showed that despite the clear inhibition in the protein expression after 18 hours of exposure to cytokine followed by VA treatment (Fig. 3A), upon blocking the protein synthesis, there is no remarkable difference in the COX-2 degradation profile in cells treated with cytokine irrespective of VA treatment (Fig. 3B, 3C and 3D). Fig. 3B indicates the level of COX-2 expression immediately after 90 minutes of cyclohexamide addition (0 hour). Figs. 3C and 3D indicate the level of COX-2 expression upon blocking the protein synthesis after 5 and 11 hours respectively. These results may indicate that the regulation of COX-2 by VA may occur in an early phase of COX-2 expression but not at the later stages of protein expression and stabilization.

Viscum album increases the COX-2mRNA degradation

Due to the indication of effect of VA in the early stages of COX-2 expression, but not at the level of its mRNA expression, we analyzed the mRNA stability of COX-2 modulated by VA. A549 cells were stimulated with IL-1β in the presence and absence of VA Qu Spez for 4 hours. After 4 hours, cells were treated with actinomycin D and harvested at different time intervals. Total cellular RNA was isolated and used for RT-PCR for the estimation of COX-2 mRNA. Treatment with IL-1β is known to induce the expression of COX-2 mRNA by transcriptional activation and also by increasing the stability of COX-2 mRNA. RT-PCR analysis of COX-2

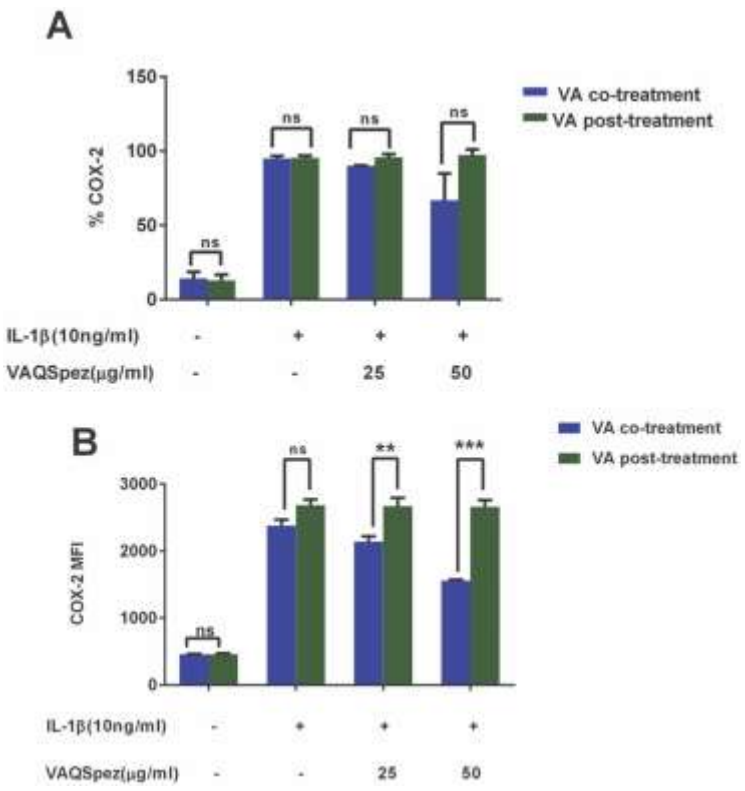

Fig 1. Co-treatment of A549 cell with IL-1β and Viscum album inhibits the cytokine-induced COX-2 expression. A549 cells were treated with IL-1β (10 ng/ml) and two different concentrations of Viscum album Q Spez preparation for 18 hours. Cytosolic COX-2 was measured using flow cytometric analysis. Viscum album is added to the cells either from the beginning of the experiment along with IL-1β (co-treatment) or after 14 hours of IL-1β induction (post-treatment). Percentage COX-2 expression as measured in intracellular staining by flow cytometry (A) and mean fluorescence intensity (MFI) of COX-2 expression (B) is shown. Results are mean ±SEM of 4 independent experiments (**p<0.01; ***p<0.001).

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mRNA expression at different time intervals after actinomycin D treatment revealed that, at any given time interval there is a tendency to decline the relative expression of COX-2 mRNA in VA-treated cells compared to the cells treated with IL-1β (Fig. 4A). This suggests that VA at 25 μg/ml increases the rate at which the COX-2 mRNA degrades in the absence of new mRNA synthesis. Further, results from RT-PCR analysis have also showed COX-2 mRNA half life, time required for 50% of the mRNA degradation in case of VA-treated cells was marginally reduced compared to that in case of cells stimulated with cytokine alone (Fig. 4B). This suggests that VA is able to reduce the mRNA half-life of COX-2 thereby leading to its reduced bioavailability for the protein synthesis.

Discussion

Prolonged administration of anti-inflammatory COX-2 inhibitors has been ineffective for chemopreventive and chemotherapeutic purposes since the risks prevail over the benefits. Clinical demonstration of severe side effects due to the failure of the classical COX-2 inhibitors to discriminate between an aberrant pathological versus homeostatic functional activation state, raised the concern that direct COX-2 enzymatic inhibition might not sufficiently represent an appropriate clinical strategy to target COX-2. Since in contrast to COX-1, COX-2 is an early response gene, similar to the genes encoded for cytokines, chemokines and proto-oncogenes,

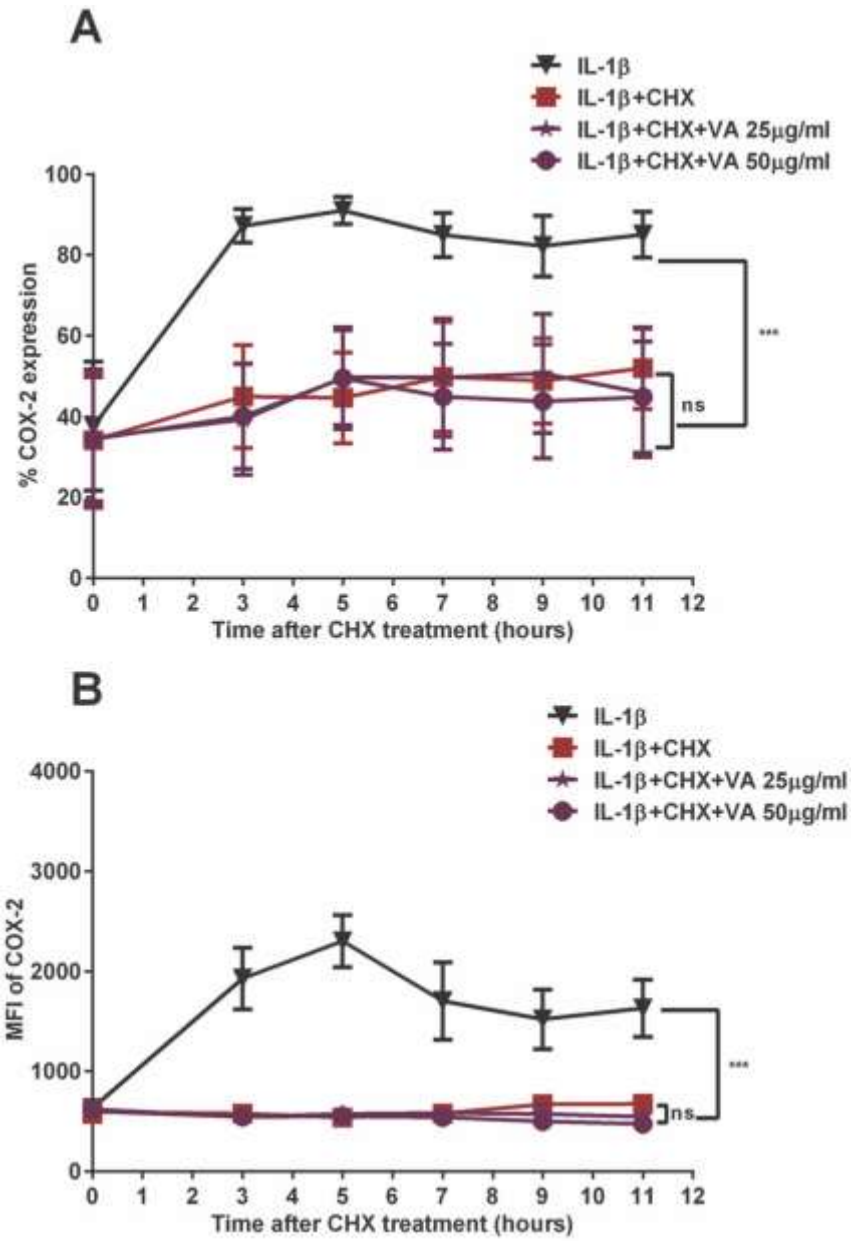

Fig 2. Effect of Viscum album on the stability of COX-2 protein as analyzed by flow cytometry. A549 cells were stimulated with IL-1β for 90 minutes with or without VA Qu Spez. Cells were harvested at different time intervals after blocking the protein synthesis with cyclohexamide (10 μg/ml) for 90 minutes till 11 hours. Normalised percentage COX-2 expression as measured in intracellular staining by flow cytometry (A) and mean fluorescence intensity (MFI) of COX-2 expression (B) is shown. Data is representative of mean ±SEM of three independent experiments.

doi:10.1371/journal.pone.0114965.g002

they can be regulated under different levels of expression and modulation, ranging from direct transcriptional effects to post-transcriptional and post-translational levels and also indirectly by various transcription factors that mediate the stability [32, 35]. Such multiple levels of modulation of COX-2 expression imply the existence of several mechanisms, which may be targeted to finely modulate COX-2 functions [36–38]. Several phytotherapeutics have been shown to exert modulatory effect on COX-2 at various levels of its molecular regulation and therefore

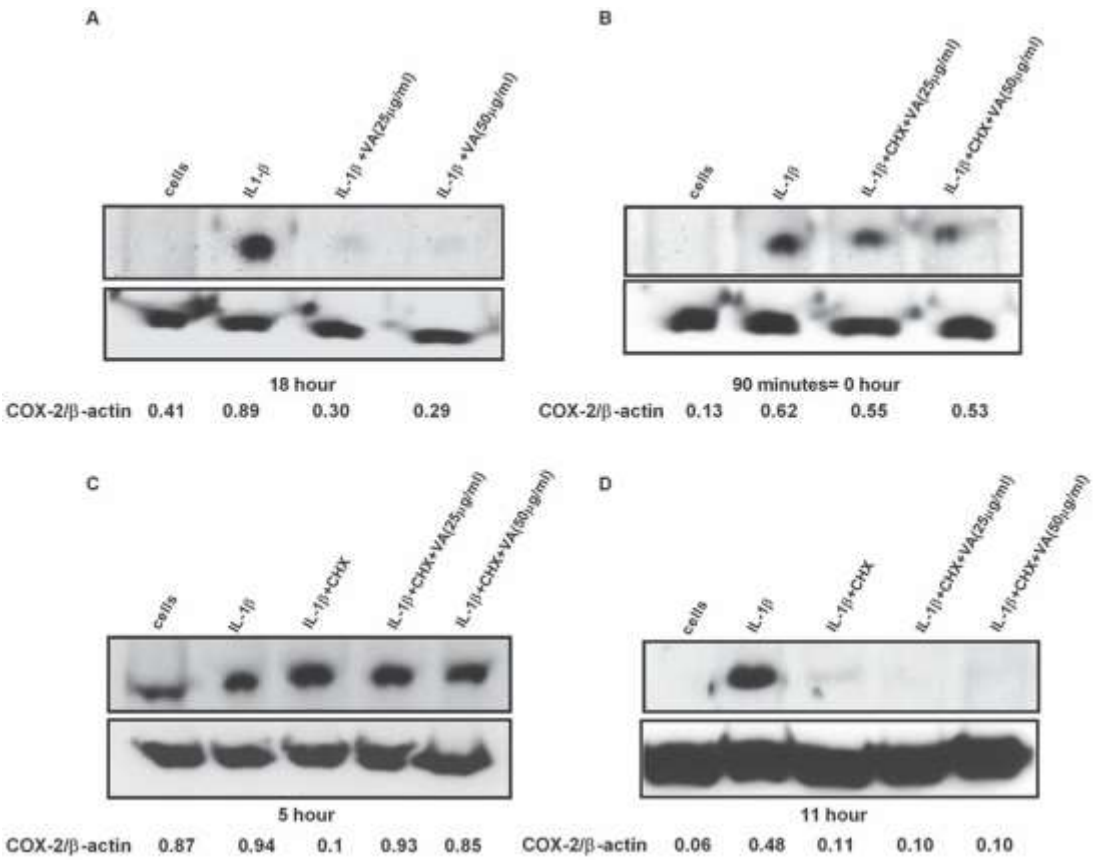

Fig 3. Effect of Viscum album on the stability of COX-2 protein as determined by western blot. Confluent A549 cells were treated with IL-1β in the presence and absence of VA Qu Spez in dose dependent concentrations in μg/ml. Cells were harvested at different time intervals after blocking the protein synthesis with cyclohexamide (10 μg/ml) for 90 minutes till 11 hours. COX-2 expression was measured by western blot using the cytosolic extracts. (A), inhibition of COX2 protein synthesis by VA at 18 hours. (B) (C) (D) are the representative western blots after 90 minutes, 5 hours and 11 hours respectively showing level of COX-2 expression after cyclohexamide treatment with or without Viscum album. β-actin was used as an internal control. All blots are representative of three independent experiments and the densitometry values for each band are mentioned below the representative blots.

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have been considered as an effective alternative strategy to control the pathogenic expression of COX-2 [33, 39, 40]. Given that VA preparations exert a potent anti-inflammatory effect by selective down regulation of COX-2, it is extremely interesting to dissect the COX-2 inhibition mediated by VA in different regulatory mechanisms at molecular level.

Co-treatment of VA along with cytokine stimulation, marginally decreases COX-2 expression indicated by the percentage-positive COX-2 expression in Fig. 1A. However, VA significantly inhibits intensity of expression of COX-2 as analyzed by MFI. The fact that VA treatment at the later phases of cytokine induction does not inhibit COX-2 suggests that, inhibition of COX-2 by VA occurs in the early phase of COX-2 regulation but not at the later phases (Fig. 1). Since we observed an inhibition of COX-2 protein expression by VA but not of mRNA, we analyzed the protein stability of COX-2 in the presence of VA by cyclohexamide pulse chase experiments. Flow cytometric analysis of COX-2 expression after 90 minutes of blocking the protein synthesis with cyclohexamide showed that, there is no significant difference in the COX-2 degradation profile of cells simulated with IL-1β with or without treatment with VA (Fig. 2A and 2B). Western blot analysis of COX-2 protein after 5 and 11 hours of cyclohexamide blockade showed no significant difference in the degradation pattern of COX-2 in cytokine stimulated cells with or without VA treatment (Fig. 3C and 3D). Similar results at

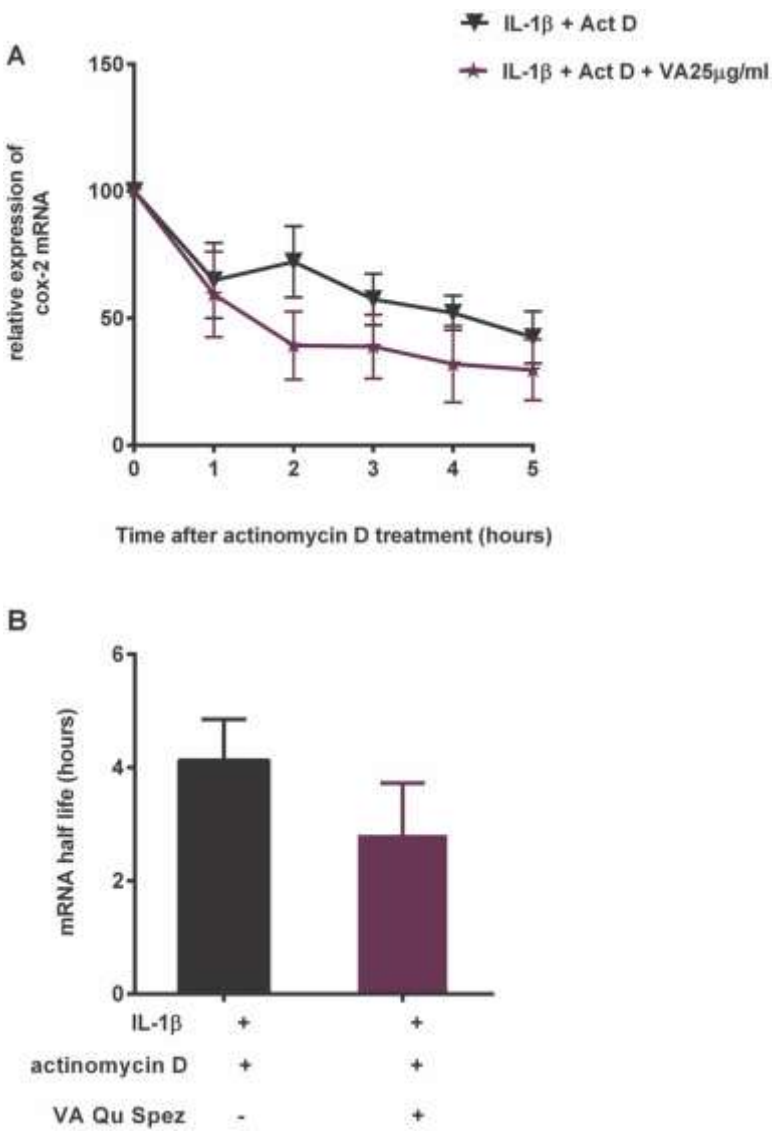

Fig 4. Increase in the COX-2 mRNA degradation by Viscum album treatment. A549 cells were stimulated with a pro-inflammatory cytokine IL-1β in the presence and absence of VA Qu Spez for 4 hours. After 4 hours of IL-1β stimulation cells are blocked with actinomycin D (10 μg/ml). Cells were harvested at different time intervals after adding actinomycin D and total cellular RNA was isolated and used for RT-PCR for the estimation of COX-2 mRNA. Relative expression of remaining COX-2 mRNA at each time point, in VA treated and untreated cells (A) and the time required for 50% of the mRNA degradation as COX-2 mRNA half life (B). Data is obtained from three independent experiments.

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different time points were observed (data not shown). Therefore, it is clear that COX-2 protein degradation is not affected by VA. Further, reduced level of COX-2 expression at 0 hour in this experiment (Fig. 3B) also suggests that, there may be modulation by VA of the COX-2 expression before the addition of inhibitor of protein synthesis. Inhibition of COX-2 protein expression by VA (Fig. 3A) without modulating its stability (Fig. 3B, 3C and 3D) strongly indicates that, there is a possible modulation by VA at an early stage than when the proteins were expressed. However VA did not modulate COX-2 mRNA expression and therefore we analyzed the mRNA stability of COX-2 by actinomycin D pulse chase experiment. mRNA degradation

profile of COX-2 obtained by analyzing the COX-2 mRNA at different time intervals after blocking the transcription using actinomycin D showed that the rate of degradation of COX-2 mRNA is higher in cells treated with VA compared to those treated with cytokine alone (Fig. 4A). This reduction in the mRNA half-life of COX-2 in the cells treated with VA (Fig. 4B) suggests that, VA induces destabilization of COX-2 mRNA, thereby diminishing the available functional mRNA for the protein synthesis and for the subsequent secretion of PGE2.

Although this study postulates destabilization of COX-2 mRNA by VA preparations as a possible mechanism for VA-mediated COX-2 inhibition, further molecular dissection is necessary in order to clearly understand the regulatory events of COX-2 regulation, contributing factors and their modulation by VA preparations.

Conclusion

Increasing body of evidence for anti-inflammatory activity of plant-derived molecules by modulating the COX-2 functions has evolved as a potent alternative strategy for the conception of novel therapeutic molecules in the treatment of various inflammatory pathologies and in various malignancies. In view of the therapeutic benefit of VA preparations in diverse pathological situations including inflammatory and cancer conditions, dissecting their molecular mechanisms would contribute enormously to the understanding of role of phytotherapy-based treatment strategies either in complementary or alternative medicine or in other combinational therapies.

Author Contributions

Conceived and designed the experiments: CS PH AF JB SVK. Performed the experiments: CS PH. Analyzed the data: CS PH AF JB SVK. Contributed reagents/materials/analysis tools: AF JB SVK. Wrote the paper: PH CS AF JB SVK.

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Differential effect of *Viscum album* preparations on the maturation and activation of human dendritic cells and CD4$^+$ T cell response

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Abstract

Extracts of *Viscum album* (VA), the semi-parasitic plant, are frequently used in complementary cancer treatment. Various reports show that VA modulates the immune system and exerts immune-adjuvant activities that might influence the tumor regression. Currently, several preparations of VA are available for the therapy. Each VA preparation is heterogeneous because of its chemical composition, which varies depending on the time of harvest, species of host tree and preparation methods; together these factors influence considerably the clinical efficacy of VA. An insight into the mechanism of action of different VA preparations is therefore necessary, that will contribute further guidelines for the utility of VA preparations in cancer treatment. In the present study we performed a comparative study involving five different preparations of VA aimed at their effect on maturation and activation of human dendritic cells (DCs) in view of the critical role they play in anti-tumoral immune response. Among five preparations tested, VA Qu Spez, a fermented extract with a high level of lectins, significantly induced DC activation, secretion of pro-inflammatory cytokines such as IL-6, IL-8, TNF-α and also enhanced the production of $CD4^+$ Th1 cytokine IFN-γ. These results are of significant relevance for adopting appropriate therapeutic regimen along with other conventional treatments.

Key words: *Viscum album; dendritic cells; cytokines; $CD4^+$ T cells; IFN-γ*

1. Introduction

Viscum album (VA), European mistletoe lectin, has been used successfully in several cancer therapies as a complementary therapy in addition to the conventional mainstream anti-tumor treatments [1]. VA belongs to the family of type II ribosome-inactivating proteins and consists of two subunits, A chain (29 KDa) and B chain (34 KDa). A-chain is responsible for ribosome inactivation whereas the B-chain helps to bind to the terminal galactoside residues on cell membrane thus enabling the entry of the protein into the cell [2,3] VA preparation is a heterogeneous mixture of several bio active molecules, but the major components of this extract are lectin and viscotoxin.

Mistletoe preparations are grown on different host trees (table 1). The difference in their biological activities majorly depends on the host trees they are derived from, the time they are harvested, and the extraction method [4,5]. Various cancer cells respond to mistletoe treatment in different manner. Lectin of this preparation is the major contributor, which is primarily responsible for the cytotoxicity of each preparation. Thus often, it is difficult to prescribe the most suitable mistletoe preparation for a specific cancer condition, as the specific response to certain preparation is not well identified. Therefore the cytotoxic effect of mistletoe is not only cell type-dependent but also dependent on the composition of each extracts. The production process considerably differs for different preparations and it is unlikely that, the effect of one particular preparation will be similar for all other extracts [6]. Attempts to distinguish the extracts using microarray analysis have revealed variations in their ability to activate several immunoregulatory genes [7].

Dendritic cells (DCs) are the versatile controllers of the immune system. They are the professional antigen presenting (APCs) and sensing cells, which are involved in initiating and modulating the immune response and bridge innate and adaptive immunity. Thus DCs are the potential targets for therapeutic intervention in immune-mediated conditions [8]. Immature DCs expressing low MHC II on their surface are specialized to internalize and process antigens. Upon interacting with specific stimuli, the DCs undergo maturation and induce T cell immunity. The mature DCs express high level of MHC II, T cell adhesive and co stimulatory molecules [9]. Under inflammatory condition, DCs receive danger signals from pathogen-associated molecules and lead to the development of effector T cells. However, in the absence of such danger signals at a steady state situation, presentation of self-antigens by

DCs results in eradication of responsive T cells or production of regulatory T cells promoting tolerance [8,10]. Whereas DCs are capable of recognizing and presenting antigens to $CD4^+$ T cells on MHC II, their ability to present the exogenous antigens to $CD8^+$ T cells on MHC I is highly regulated and this cross-presentation is important for tumor regression for generation of cytotoxic T lymphocytes [11,12].

When the host anti-tumor immune response is compromised, tumors can evade immunosureveillance. DCs are the central players to induce anti-tumor immune responses and requisite function of these cells is crucial for the success of the cancer immunotherapy [13]. DCs are immature and functionally defective in cancer patients and tumor-bearing animals. Functional impairment of DCs is the repercussion of the insufficient danger signal in tumor environment [14]. The major reason for functional inability of DC could be direct contact of immature DCs to tumor cells that hamper maturation process of DC and antigen presentation to T cells. [15-18].

There is strong consistent evidence of relevant therapeutic efficacy of mistletoe in the field of cancer to improve survival of patients, damage recovery caused by the conventional cancer therapies and to improve the quality of life of the patients [19,20] Further, VA-educated DCs favour them towards maturation and activation [21]. Clinical outcome of one preparation is not necessarily similar for other preparations because of the variation in the composition [22]. Thus the effects of VA have to be ideally authenticated by means of controlled trials for every single VA preparation. In the current study, we thus set out to investigate the 'potent' VA preparation among several commercially available preparations from immunomodulatory angle by exploring their differential effect on DC activation.

2. Materials and Methods

2.1. VA preparations

Five clinically validated preparations of VA namely VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A were a kind gift from Weleda AG (Arlesheim, Switzerland). These are the extracts of VA that grow on different trees like oak, apple, pine and abies. All these preparations are free from endotoxins. VA preparations are formulated in 0.9% sodium chloride isotonic solution as 5 mg/ml vials. During the manufacturing process, VA preparations are prepared by standardizing the levels of mistletoe lectins and viscotoxins and the method of preparing these standard extracts are different, thus they are either fermented or unfermented (see: Table1).

Table1. Composition of VA preparations

Preparation Concentration	Host trees	Lectin Content (ng/ml)	Viscotoxin content (µg/ml)	Method of preparation
VA Qu Spez 10 mg	Quercus (Oak)	785±10%	5±5%	Fermented
VA Qu Frf 10 mg	Quercus (Oak)	2391±10%	19±5%	Unfermented
VA M Spez 10 mg	Malus (Apple)	548±10%	4±5%	Fermented
VA P 10 mg	Pinus (Pine)	28±10%	6±5%	Fermented
VA A 10 mg	Abies (Fir)	23±10%	19±5%	Fermented

2.2. Differentiation of human monocyte-derived DCs

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors obtained from Centre Necker-Cabanel (EFS, Paris). Circulating monocytes were isolated using CD14 beads (MiltenyiBiotec, France) and were cultured for 5 days in RPMI 1640 containing 10% FCS, rhIL-4 (500 IU/10^6 cells) and rhGM-CSF (1000 IU/10^6 cells) to obtain immature DCs as previously described [23].

2.3. Viscum album treatment of DCs

Immature DCs were washed and cultured in respective cytokines and treated with VA Qu Spez, VA Qu Frf, VA M Spez, VA P and VA A at four different concentrations: 5, 10, 15 and 20 µg/ml for 48 hours. Cell culture supernatants were collected for analysing cytokines and the phenotype of cells was analyzed by flow cytometry.

2.4. DC: $CD4^+$ T cell co-cultures

$CD4^+$ T cells were obtained from PBMC using CD4 microbeads (MiltenyBiotec). DCs following treatment with VA were washed extensively and seeded with 1×10^5 responder allogenic $CD4^+$ T cells at DC:T cell ratio of 1:10. Post 5^{th} day, cell culture supernatants were collected for analysing cytokines and cells were analyzed for either intracellular T-cell cytokines/transcription factor.

2.5. Flow cytometry

FITC-conjugated monoclonal antibodies (MAbs) to CD1a, CD86, HLA-DR, and CD25; PE-conjugated MAbs to CD83, (BD Biosciences, France), CD40 (Beckman Coulter, France) and Alexa Flour®700-conjugated MAbs to CD4 (eBioscience, France) were used for the surface phenotype analysis.

For intra-cellular staining, FITC-conjugated MAbs to IFN-γ (eBioscience), PE-conjugated MAbs to IL-17A and IL-4 (eBioscience) and APC–conjugated MAbs to Foxp3 (eBioscience) were used. Live-dead cells were differentiated by PO-Fixable Viable Dye (eBioscience).

For surface staining, following Fc receptor blockade, antibodies against surface molecules were added at pre-determined concentration and incubated at 4°C for 30 min. Cells were acquired on LSRII and processed with FACS DIVA software (BD Biosciences) and analysed by Flowjo. The data are presented as % positive cells for indicated markers or mean fluorescence intensities (MFI) of their expression.

For intra-cellular staining, cells were stimulated with phorbolmyristate acetate (50 ng/ml; Sigma-Aldrich, France) and ionomycin (500 ng/ml; Sigma-Aldrich) at 37°C for 5-6 hours in the presence of golgistop (BD Biosciences) during the last 2 hours. Cells were fixed and permeabilized using Foxp3 Fixation/Permeabilization kit (eBioscience) and incubated at 4° C with anti-Foxp3.

2.6. Cytokine assay

IL-6, IL-8, IL-10, TNF-α, IL-4, IL-13, IFN-γ and IL-17 in cell-free culture supernatants were quantified by Ready-SET-Go enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, France).

2.7. Statistical analysis

The significant difference between samples were determined by One way ANOVA Tukey's Multiple Comparison Test using Prism 5 software (GraphPad Software, Inc, La Jolla, Calif). Values of $P<0.05$ were considered statistically correlated (*$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$).

3. Results

3.1. Comparison of the effect of different VA preparations on the maturation of DCs

Aim of this study was to investigate the differential effect of five VA preparations on immature human DCs. Immature DCs of 5 day old culture were either untreated or treated with five VA preparations and each at four different concentrations: 5 µg/ml, 10 µg/ml, 15

μg/ml, 20 μg/ml for 48 hours. Post two days, cells were analyzed for the expression of several surface molecules (Fig. 1A-F). VA Qu Frf, VA M Spez, VA P, VA A were not able to enhance the expression of antigen presenting molecule HLA-DR and co-stimulatory molecules such as CD80, CD86, CD83 and CD40. Interestingly, VA Qu Spez was able to enhance the expression of both antigen presenting molecules and the co-stimulatory molecules. These results suggest that among all the preparations tested; only VA Qu Spez is able to induce maturation of DCs.

3.2. Comparison of the differential effect of VA preparations on the secretion of DC cytokines

It is well reported that DC-derived cytokines are crucial for priming T-cell response. We analyzed the differential effect of five VA preparations on the level of secreted cytokines such as IL-6, IL-8, IL-10 and TNF-α. As shown in Fig.1, VA Qu Spez was capable of activating DCs, in turn suggesting the possible effect of VA Qu Spez on the secretion of cytokines. Compared to untreated DCs, VA Qu Spez-treated DCs showed significantly increased secretion of IL-6, IL-8 and TNF-α (Fig. 2A, B, D). The untreated or control DCs secreted 4.773 ± 5.17 pg/ml of IL-6 and was significantly enhanced to 156.957 ±105.15 pg/ml by VA Qu Spez. In case of IL-8, the control DCs secreted 102.29 ± 78.54 pg/ml, whereas VA Qu Spez at highest concentration, induced 612.13 ±20.47 pg/ml. TNF-α secretion by untreated DCs was 3.22 pg/ml, and with VA Qu Spez-treatment, this cytokine was increased to 135.7 ± 37.9 pg/ml. Apart from VA Qu Spez, even VA Qu Frf and VA M Spez showed moderate enhancement of above pro-inflammatory cytokines, but VA P and VA A were unable to modulate DC cytokines. These results showed that VA Qu Spez is the most potent preparation capable of enhancing IL-6, IL-8 and TNF-α. IL-10 level was unaltered with all five VA treatments even at highest dose (Fig. 2C). Together, our data suggest that VA Qu Spez significantly induces several pro-inflammatory cytokine secretions without modulating immune-suppressive cytokine IL-10.

3.3. Comparison of the effect of VA preparations on the $CD4^+$ T cell response

One of the key functions of APC is to promote T cell response. DCs primed with various preparations of VA were co-cultured with allogenic total $CD4^+$ T cells at a ratio of 1:10 and Th1, Th2, Th17 and T regulatory cells (Treg) responses were measured by flow cytometric analysis of intracellular IFN-γ (Th1), IL-4 (Th2), IL-17 (Th17), FOXP3 (Treg). Although VA Qu Spez induced maturation of DC, this effect was not associated with the modulation of frequency of various T cell subsets (Fig. 3A-D). However, analysis of T cell cytokines in DC-

T cell co-culture revealed that VA Qu Spez significantly stimulated IFN-γ secretion (Fig. 4A), without having any effect on the secretion of IL-4 (Fig.4B), IL-13 (Fig. 4C) and IL-17 (Fig. 4D). As expected, other four preparations of VA did not alter either frequency of T cell subsets or secretion of T cell cytokines. Taken together, these results suggested that VA Qu Spez favours Th1 response.

4. Discussion

IFN-γ plays an important role in mediating protective immune response against cancer cells and viral and intracellular bacterial infections [24]. IFN-γ enhances MHC class I expression on tumor cells and MHC class II expression on antigen presenting cells like DCs, which in turn link innate and adaptive immunity [25]. IFN-γ responsiveness of the tumor cell is important for successful immune recognition; as it was demonstrated that mice, which were non-responsive to IFN-γ develop more tumors compared to wild-type mice. Studies show that cross-talk between lymphocytes and IFN-γ/STAT1 signalling pathway, plays an important role in maintaining the immune competiveness of the host in the fight against tumors [26]. Tumor apoptosis can be achieved by idiotype specific $CD4^+$ Th1 cell directly by Fas/Fas L interaction and indirectly by IFN-γ production, which can regress tumor [27]. Thus, IFN-γ is important in forming the basis of an extrinsic tumor-suppressor mechanism [28]. VA significantly enhanced IFN-γ, which strongly supports the fact that VA is an immune modulator [29].

Several DC-based cancer immunotherapies have been established with the aim of enhancing DC maturation with high migration capacity and enhancing tumor suppressive mechanisms mediated by CTLs [8,30]. There are reports suggesting that IFN-γ enhances apoptotic response to mistletoe lectin (ML) II through enhancement of Fas/Fas L expression and caspase activation in human myeloid U937 cells [31]. Our data demonstrates, among various VA preparations tested, VA Qu Spez is able to enhance the expression of antigen presenting and co-stimulatory molecules on human DCs. Furthermore, VA Qu Spez-educated DCs co-cultured with allogenic $CD4^+$ T cells were able to secrete significant amount of IFN-γ, suggesting VA drives Th1 response, which could manifest the anti-tumoral immune response of VA along with its immunomodulatory response in the host.

Currently available mistletoe extracts are highly heterogeneous preparations because of the difference in their host trees, nutritional source, time of harvest, and the method of extraction [4,5], thus different preparations could exert divergent biological activities. However, biological effects of different preparations of VA extracts have not been assessed till date. In particular, analysis of the effect of different VA preparations on immunocompetent cells such

as DCs and its correlation with the lectin content has not been reported. The present comparative study of different VA preparations represents an important aspect in the field of phytotherapy to be addressed in order to understand the underlying mechanism of action of VA.

MLs are the active components of mistletoe extracts and have several functions. MLs are responsible for stimulating effector cells of the innate and adaptive immune system such as DCs, macrophages, natural killer cells, and B and T lymphocytes. This function of MLs might represent one of the mechanisms responsible for the anti-tumoral effect of mistletoe extracts. It is known that ML-I B-chain (MLB) causes Ca^{2+} influx in Jurkat cells mediated by its interaction with surface glycoprotein receptors which participates in early activation of T-cells [32]. Chemical labelling of the lectin revealed that it binds to surface of peripheral and intratumoral monocytes and this lectin component of ML-I plays a major role in immunomodulation [33]. Depending on the concentration used for treatment, mistletoe extracts also induce cell death in tumor cells and exert direct necrotic effects or apoptosis [34]. Cytotoxicity of mistletoe is attributed majorly to its lectin contents [35, 36]. Several studies have clearly demonstrated that lectin internalization is required for ML-I mediated apoptosis, independent of surface receptor-mediated pathway [37].

VA Qu Frf, an unfermented preparation containing highest concentration of lectin and viscotoxin, was unable to activate DCs. In parallel, other VA preparations, which are fermented and contain low lectin, were unable to stimulate DCs. Whereas VA Qu Spez, a fermented preparation which contains second highest concentration of lectin, (785 +/- 10% ng/ml) efficiently activated DCs and promoted Th1 response. These results suggest that, in addition to the lectin content, the methodology of preparation, i.e., fermented vs unfermented is crucial in conferring stimulatory properties to VA and indicates that it might be possible that fermentation could have an effect on the lectin structure.

A recent study shows that the 3D structure of ML-A chain shares structural homology with shiga toxin from *Shigella dysenteriae*. As Mistletoe plant produces a bacterial toxin, it has strong immune stimulatory capacity [38]. It is also demonstrated that the Korean mistletoe lectin (KML) is a TLR-4 ligand [39]. Since Korean ML and European ML have 84 % of sequence identity [40], it is presumable that european ML could mimic TLR-4 ligand [38]. It is known that TLRs are potent activators of DCs, which is a pre-requisite to initiate a full-blown T cell response against cancer. Several clinical studies have provided evidence in support of the beneficial effects of mistletoe in cancer patients and mistletoe thus remains as one of the remedies most often used as a complementary therapy.

Taken together, our study delineates the differential effects of various VA preparations and reveals VA Qu Spez to be the potent preparation in activating DCs and promoting Th1 response. Thus, this study along with other reports provides a rational for the use of VA Qu Spez as an immune modulator [41-43] and further strengthens the beneficial effect of VA preparations as complimentary therapy in cancer.

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Figure Legend

Fig 1. Comparison of the differential effects of VA preparations on phenotype of human DCs**.** Immature DCs were treated with medium alone (control) or with 5 preparations of VA at various concentrations for 48 hours. Expressions (mean ±SEM, ≥ 5 independent donors) (A) CD1a, (B) CD83, (C) HLA-DR, (D) CD40, (E, F) CD86 on DCs were analyzed by flow cytometry. The data are presented either as % positive cells or MFI of indicated markers. X-axis denotes concentrations of VA preparations. *p <0.05, ***p <0.001**.**

Fig 2. Comparison of the effect of VA preparations on the secretion of DC cytokines**.** Immature DCs were untreated (control) or treated with 5 preparations of VA at various concentrations for 48 hours. The secretion (pg/ml, mean ± SEM, four independent donors) of (A) IL-6, (B) IL-8, (C) IL-10 and (D) TNF-α in cell-free supernatants were measured. **p< 0.01, ***p < 0.001, ****p <0.0001.

Fig 3. Differential effect of VA preparations on T cell response. DCs treated with medium alone (control) or with 5 preparations of VA for 48 hours. These DCs were co-cultured with allogenic $CD4^+$ T cells at 1:10 ratio. After 5 days of co-culture, the cells were analyzed. A-D indicates percentage (mean ± SEM, six independent donors) of IFN-γ^+ Th1, IL-4^+ Th2, IL-17^+ Th17 and $CD25^+Foxp3^+$ Treg cells. ns, non-significance.

Fig 4. Effect of VA preparations on T cell cytokine secretion. Immature DCs were treated with medium alone (control) or with 5 preparations of VA for 48 hours. These DCs were co-cultured with allogenic $CD4^+$ T cells for 5 days. Amount of secretion (pg/ml, mean ± SEM, six independent donors) of (A) IFN-γ, (B) IL-4, (C) IL-13 and (D) IL-17 in the cell-free supernatants from the above co-cultures. *p< 0.05.

Figure1.

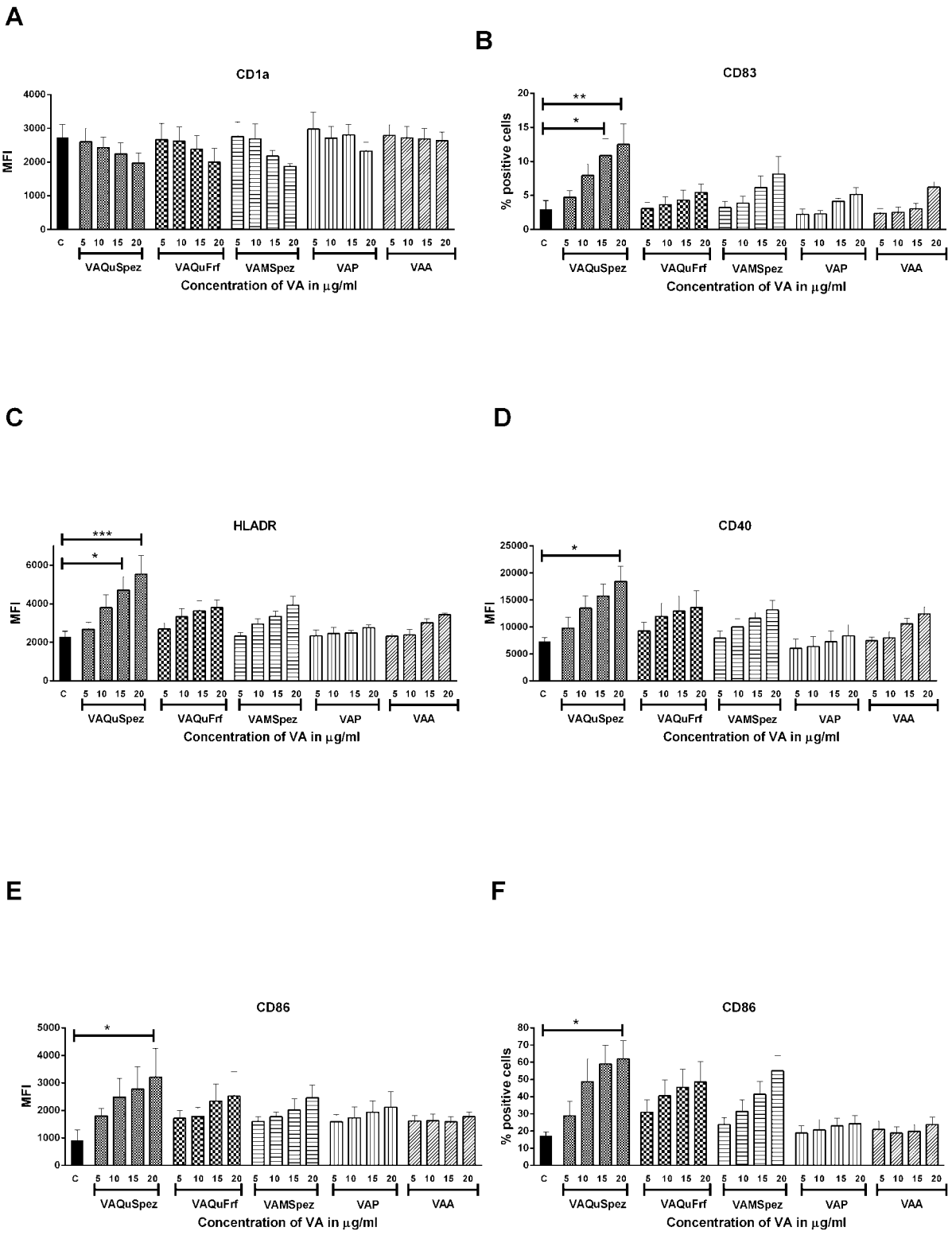
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Figure2.

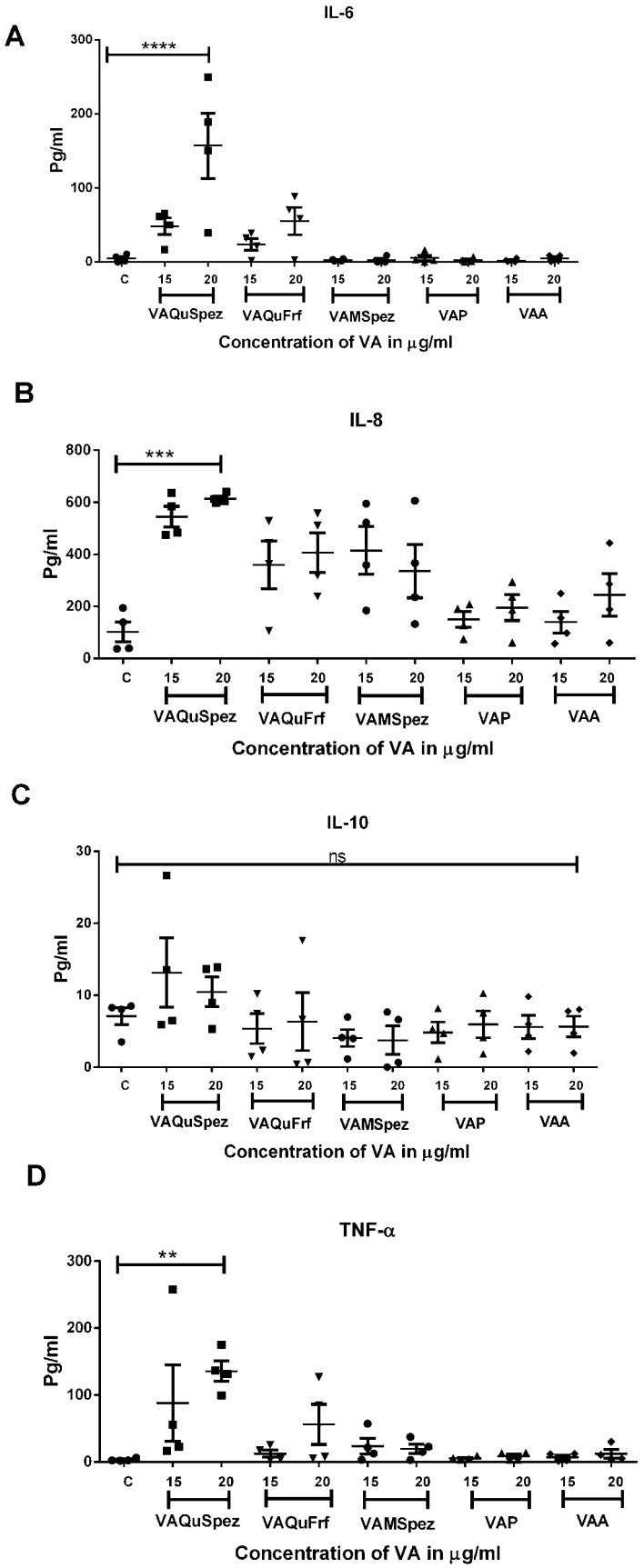
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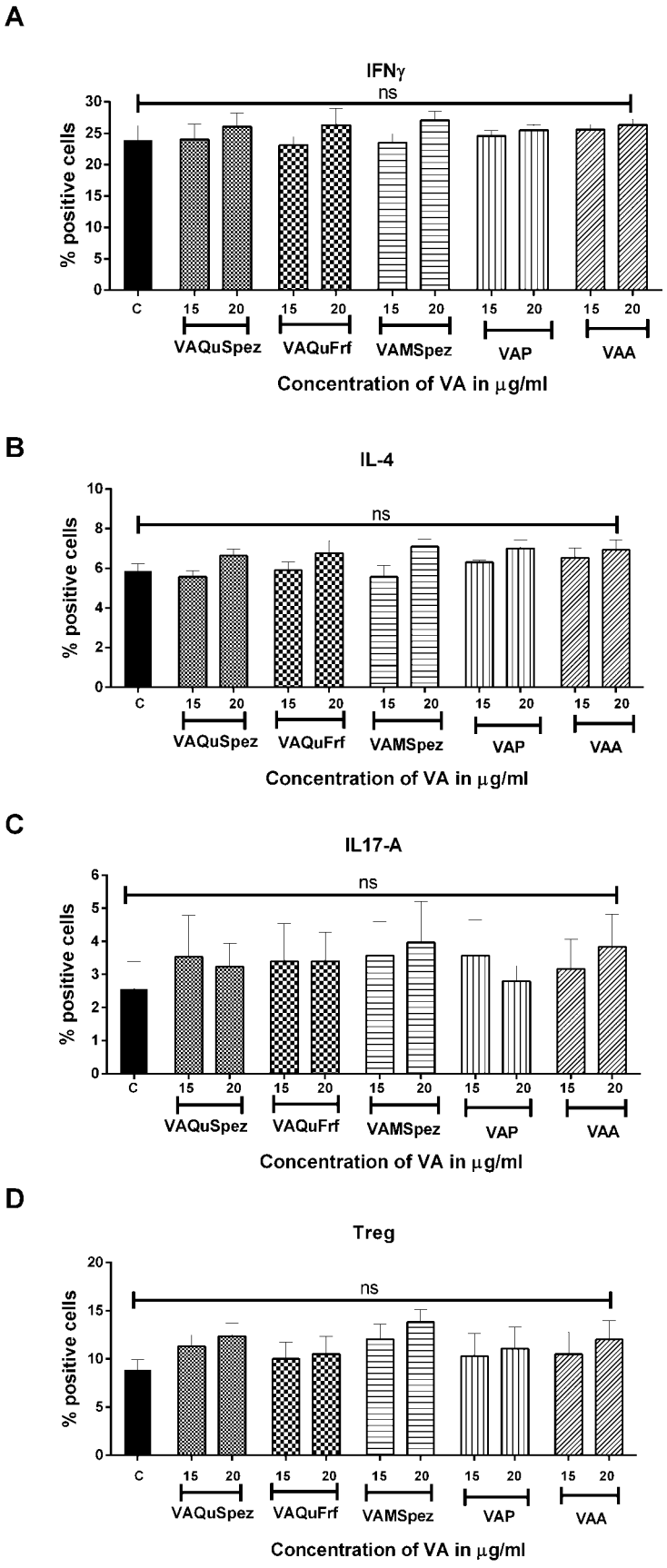
Figure3.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Concentration of VA in µg/ml
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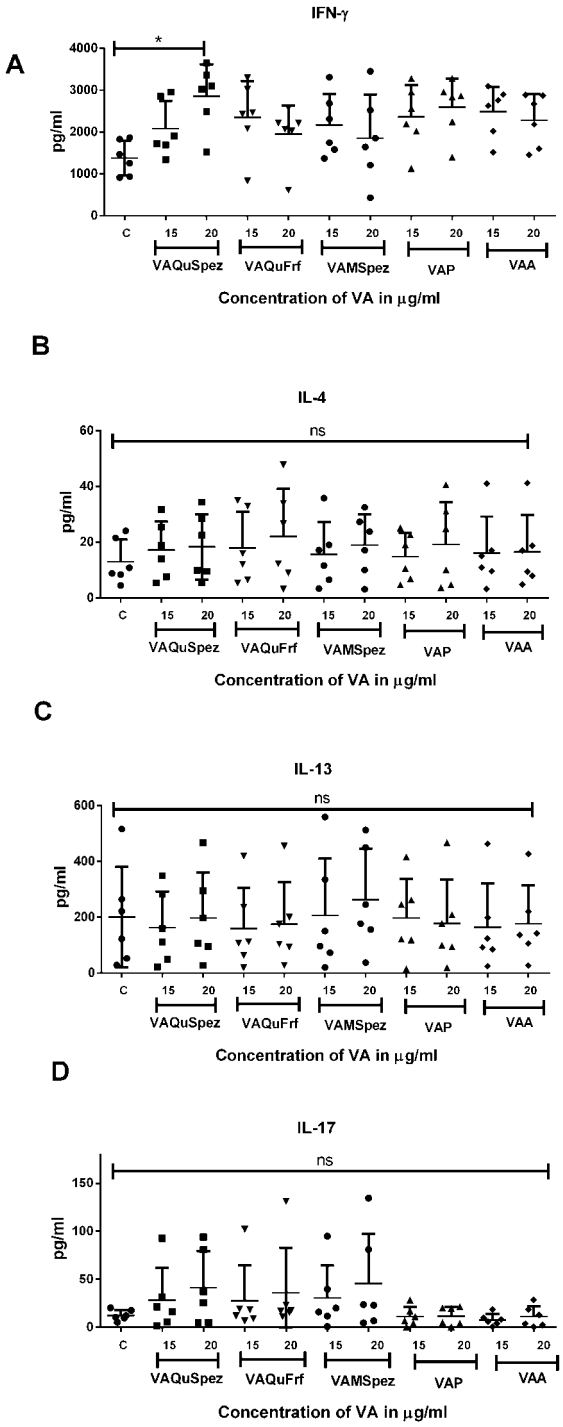
Figure4.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Concentration of VA in µg/ml

Viscum album promotes anti-tumor response by modulating M1/M2 macrophage polarization switch

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Abstract

Wide use of *Viscum album* (VA) preparations in complementary and alternative therapies for cancer has revealed their beneficial effects on the survival and the quality of life of cancer patients. The underlying molecular and cellular mechanism of their therapeutic efficacy encompasses cytotoxic properties, inhibition of angiogenesis and importantly several immunomodulatory functions. Tumor progression depends on their ability to re-educate the polarization state of tumor associated macrophages from pro-inflammatory M1 to anti-inflammatory M2. The orchestration of monocyte-macrophage function is a key element that links inflammation and cancer and provides a paradigm for macrophage diversity and plasticity. Thus, we have investigated whether VA can modulate macrophage polarization, which in turn can be associated with their anti-tumor property. Our data demonstrates VA mediates the switch of M2 macrophage polarization towards M1 macrophages. Therefore, the immunomodulatory activity of VA is dependent on the polarization state of the responding macrophages, and their ability to drive M1 macrophages over M2 macrophages might be another angle underlying their therapeutic benefit in cancer.

Key words: *Viscum album, myelomonocytic cells, macrophage cells, Immune dysregulation, pro-inflammatory cytokines*

Introduction:

A constant influx of myelomonocytic cells is an important characteristic of tumor to support stroma remodelling and angiogenesis for their continuous growth. In early 1980s an interesting population called *natural suppressors* was identified which are known to be associated with immune suppression and tumor development [1]. Another fascinating observation was in tumor microenvironment, number of myeloid cells are elevated which in turn alter anti-tumor immune reactivity [2], [3]. Immune dysregulation and immune suppression are the common features in cancer bearing patients in which even tumor-specific T cell death can occur [4]. At tumor site, accumulation of immunosuppressive myelomonocytic cells can take place [5], [6]. Recently these immune suppressor cells are called as myeloid-derived suppressor cells (MDSCs), which are deficient of expressing mature myeloid cell markers [7]. In 1980s, Alberto Mantovani originally described that [8], in the tumor site, circulating monocytes are recruited and with the help of a tumor-derived chemotactic factor CCL2, these cells differentiate into tumor associated macrophages (TAMs) [9]. These tumor-derived chemokines along with recruiting monocytes in the tumor site play an important role in tumor progression by encouraging inflammation and angiogenesis thus inducing neoplastic growth [10]. TAMs are the second population of myelomonocytic immune suppressors which shows a negative effect on anti-tumor immune responses, they are believed to be derived from or related MDSCs [4]. Cancer-related inflammation is associated with mononuclear phagocytes [11], [12] and cancer is a major paradigm of macrophage (M diversity and plasticity [13], [14], [15].

Considering T helper type 1 (Th1) and T helper type 2 (Th2), polarization, two distinct types of macrophages have been recognized: classically activated macrophages-M1 and alternatively activated macrophages-M2 [16], [13]. Th1 cells can drive M1 macrophages by producing IFN-γ and even bacterial moieties such as LPS can polarize M1 macrophages. These cells are characterized by their ability to secrete pro-inflammatory cytokines such as IL-12, IL-23 and tumor necrosis factor (TNF), reactive oxygen and nitrogen species, antigen presentation and elevated expression of major histocompatibility complex II, and tumoricidal activity [17]. Through expression of several cytokines and chemokines such as IL-12, CXCL-9 and CXCL-10, they recruit Th1 cells thus promoting Th1 response [9]. They confer resistance to intracellular pathogens and tumors leading to tumor-disruptive response [16], [18]. M1 macrophages show a phenotype of high IL-12 and low IL-10 expression. In contrast, Th2 cytokine IL-4 triggers M2 macrophage polarization [19]. These cells are involved in amplifying Th2 responses. These cells are more phagocytic in nature and exert high

expression of scavenging, galactose and mannose receptors, through arginase pathway they produce polyamines and ornithine, and even show a characteristic of expressing low IL-12 and high IL-10 [16], [13], [18]. M2 macrophage expressing chemokines are CCL17, CCL22 and CCL24 [20]. As M2 macrophages drive Th2 immune response in turn they actively participate in encapsulation and clearance of parasites hence in tumor progression and tissue remodelling and importantly exert an immune regulatory property [17].

Viscum album (VA) commonly known as European mistletoe is extensively used in complementary and alternative medicine in cancer and also in the treatment of several inflammatory diseases [21]. Several systemic reviews and meta-analyses supports a beneficial effect of mistletoe treatment on cancer patient survival [22], [23] and due to their ability to improve quality of life of cancer patients [22], [24], [25] and minimising the side effects of conventional anti-cancer therapies [24]. Viscum preparations consist of multiple components at variable concentrations, depending on the host tree they are harvested from, the time at which they were harvested and the method of preparation. Mistletoe lectins (MLs) I-III, especially ML-I and viscotoxins are the major bioactive components of this preparation which are majorly involved to exert their anti-cancer property [26]. This preparation also composed of several other biologically active components such as flavonoids, peptides, several enzymes, polysaccharides, phenols, triterpenes, lipids, phytosterols, amino acids, thiols, cyclitoles, phenylpropanes and minerals [27]. ML-I belongs to a class of ribosome inactivating protein (RIP), such as highly toxic ricin and abirin [28]. It consists of two subunits, the α and the β-chain connected by a disulphide bridge [29]. The β-chain binds to sugar components expressed on the surface of cells which enables their uptake into the cells. This characteristic of viscum is believed to be the reason for their specific anti-cancer effects. The α-chain exerts RNA-glycoside hydrolase activity, resulting in inactivation of ribosomes hence their ability to induce apoptosis [30]. Increasing evidence has revealed that VA exerts anti-tumor activities including cytotoxic properties [31], [32], induction of apoptosis [33], inhibition of angiogenesis [34], and immunomodulatory properties and anti-inflammatory properties [35], [36].

There is increasing evidence supporting the fact that in cancer, the M2-like pro-tumoral phenotype of TAMs is reversible [37], [38]. Guiducci *et al*. reported that CpG activated TLR9 and IL-10 Ab can switch M2 TAMs to M1 phenotype [39]. Notch signalling can enhance anti-tumor activity by encouraging classically activated macrophages, which are known to kill cancer cells [40]. Re-directing macrophage polarization can be achieved by genetic blocking of molecular determinants of macrophage polarization such as STAT3, STAT6, NF-kB p50

which might lead to anti-tumor activity [37], [41], [42]. Thus it is confirmed that in early stages of cancer T cell response decides classical macrophage polarization. Therefore, in view of the anti-tumoral and immunomodulatory effect of viscum, we were interested to investigate whether VA can modulate macrophage polarization, which in part explains the mechanisms underlying the beneficial effect of VA in cancer therapy.

Results

VA Qu Spez induced switch of macrophage polarization in a M1/M2 mixed phenotype

We first investigated whether VA modulates polarization of M1 and M2 macrophages. M1 macrophages are known to be pro-inflammatory in nature, thus we were initially interested to study the effect of VA Qu Spez on polarization of M1 macrophages. The cells were cultured either with GMCSF, LPS and IFN-γ to obtain M1 macrophages or with M-CSF, IL-13 and IL-4 for M2 macrophage polarization. In both the culture conditions we obtained a mixed M1/M2 phenotype population of macrophages. And in both the conditions, interestingly VA Qu Spez was able to significantly favour M1 phenotype over M2 phenotype. VA induced M1 phenotype is showed by elevated expression of M1 surface markers such as CD80 (Fig. 2B and 2C) and CCR7 (Fig. 2D). Along the same line VA provoked a dramatic polarization switch in M2 macrophages, as it reduced the expression of M2 specific marker CD206 (Fig. 1C). Altogether these results demonstrate the ability of VA Qu Spez to promote M2 to M1 polarization switch in human macrophages.

Significant induction of IL-12 expression by VA Qu Spez in a M1/M2 mixed phenotype indicating the effect of VA in M2 to M1 polarization switch

M1 macrophages are characterized by high expression of IL-12 and low IL-10 expression, but in contrast, M2 macrophages exert a phenotype of low IL-12 and high IL-10 expression. Previous results indicated that VA is able to favour M1 macrophage polarization over M2. Thus we were interested to explore whether VA can modulate the expression of IL-12 and IL-10, which in turn can strengthen our previous observation. Interestingly in both the culture condition of M1 and M2, VA Qu Spez significantly induced IL-12 expression (Fig. 3A and 3D), confirming that VA skew them towards the acquisition of the phenotypic characteristics of M1 macrophages.

VA Qu Spez significantly drives M1 polarization in distinct M2 macrophage population

To determine the extent of the relevance of these findings, we next determined whether VA Qu Spez-mediated polarization switch in a mixed M1/M2 phenotype, holds true even in a

distinct M1 and M2 scenario. Thus we validated polarization state of distinct M1 and M2 macrophages. Macrophages showing significant induced expression of CD80 and CCR7, confirmed specific M1 population (Fig. 4A), whereas elevated expression of CD206 and CD209 validated specific M2 polarized macrophages (Fig. 5A). Although there was no modulation of polarization observed with VA Qu Spez treatment in both M1 and in M2 cells (Fig4. and Fig5. B-E), but surprisingly induced CD80 expression by VA treatment compared to the untreated cells in M2 macrophages, once again strongly indicates the role of VA in switching M2 to M1 phenotype.

VA Qu Spez drives M2 to M1 switch by inhibiting expression of IL-10 in M2 macrophages

Regarding previously reported immunomodulatory and anti-inflammatory properties of VA Qu Spez and the prognostic impact of alternatively polarized macrophages, we were interested in the effect of VA Qu Spez on polarization of macrophages. Macrophages were polarized *in-vitro* as described above to obtain distinct M1 and M2 phenotype. There was no significant alteration in the IL12 and IL-10 profile (Fig. 6A, 6b and 6D) after treatment with VA compared to the control untreated cells. But interestingly VA significantly abrogated the expression of IL-10 in M2 macrophages. Therefore all results collectively suggest that, VA Qu Spez alters the macrophage polarization, i.e., drives alternatively activated macrophage switch towards classically activated macrophages, which in turn can be beneficial to limit tumor growth.

Discussion

Mechanisms of immune surveillance are able to control the growth of new tumors or affect the progression of existing tumors is well known [43]. Immune dysregulation and immune suppression in cancer patients is a composite event which in turn leads to abnormal myelopoesis and recruitment of several immunosuppressive myelomonocytic cells at tumor vicinity [5], [6]. This immune dysfunction even can cause death of tumor-speecific T cells or lymphocyte dysfunction. These immunosuppressive myelomonocytic cells are involved in process of angiogenesis and stroma remodelling needed for tumor progression. In the tumor site, tumor-derived factors are involved in functional differentiation of myelomonocytic cells majorly the macrophages to sustain myelopoiesis [4]. Regulation of Monocyte-macrophage function at equilibrium is essential for dealing with pathogens, tissue damage and repair. The orchestration of myelomonocytic cell function links inflammation and cancer. Macrophages show considerable functional plasticity and exert an immediate response to alter according to

the change in the microenvironment [44]. Cancer serves a paradigm of macrophage plasticity and diversity [13], [14], [15]. Based on Th1 and Th2 polarization, macrophages are classified in two distinct states: the classically activated macrophages or M1 and the alternatively activated macrophages or M2 [17]. Myelomonocytic cells are involved in carcinogenesis at every possible stages of tumor progression [11], [12], [45]. These cells influence the following steps: suppressing adaptive immune response [46], supporting angiogenic network and lymphangiogenesis [47], [48], [49], promoting genetic instability, senescence regulation [50], promoting invasion and tumor metastasis [48]. But on the other hand tumor cells are capable of escaping phagocytosis by macrophage cells [51], and majorly by recruiting M2 macrophages in the tumor site through chemokines and cytokines such as MCSF, IL-10, TGF-β etc. [52], [11].

Several study showed that *Viscum album* exerts anti-tumor properties. VA induces cell toxicity and inhibits proliferation of a variety of cell types [31], VA Qu Frf mistletoe extract induces apoptosis in a variety of transformed cells in a Fas independent way [32]. Kaveri group had demonstrated anti- tumoral property of that VA and reported that VA is able to induce apoptosis of endothelial cells and can inhibit angiogenesis which is essential for tumor metastasis [33], [34]. Apart from its anti-tumor properties VA also employs a novel anti-inflammatory effect by inhibiting cytokine induced prostaglandin E2 via selective inhibition of Cyclooxygenase-2 [53], [54]. In addition to its ani-tumor properties, viscum preparations have immunomodulatory effects. VA facilitates tumor elimination in experimental models [55]. VA lectin induces gene expression of IL-1α, IL-6, TNF-α, IFN-γ and GM-CSF from PBMC [56]. VA preparations stimulate the maturation and activation of human DCs, which in turn might facilitate anti-tumor immune responses [36]. Triterpenes in VALE is shown to have immunomodulatory effects on tumor cell co-cultured macrophages *in vitro* by modulating monocyte chemotactic transmigration [57]. Mistletoe exerts a beneficial effect on cancer patient survival [22], [23] and their ability to improve quality of life of cancer patients [24], [25] and minimising the side effects of conventional anti-cancer therapies [24] is demonstrated by several systemic reviews and clinical studies.

In the present *in vitro* study, we demonstrate that VA Qu Spez drives M2 switch towards M1, in a mixed M1/M2 population as well as in distinct M1 and M2 phenotype. VA- mediated modulation of this switch is demonstrated by induced expression of M1 surface markers such as CD80 and CCR7, but in parallel, reduced expression of M2 surface markers such as CD206 and CD209, with VA treatment in both the culture conditions. In addition, VA provoked polarization of IL-12^{hi} and IL-10^{lo} characterised M1cells over IL12^{lo} and IL-10^{hi} M2

macrophages, suggesting that VA is able to play M2 to M1switch. There are several reports suggest that M2-like pro tumoral phenotype of TAMs in cancer is reversible [38] [58]. Tumor associated macrophages are the main source of IL-10 which is an imuunomodulating cytokine which can turn down innate and adaptive immune response and they are responsible to maintain M2 state of macrophages [39]. Strategies to overcome tumor escape mechanisms by changing the immunosuppressive situation are the key to effective immunotherapy. Our results show that VA down modulates IL-10 expression in M2 macrophages will probably be one of the reasons for VA to exert immunomodulatory effect. IL-12 links innate and adaptive immunity by promoting cytotoxic T lymphocytes (CTL). Reduced production of CTL and abrogation of tumor rejection was observed in IL-12 knockout mice [39]. In this study we show that VA Qu Spez was able to significantly induce IL-12 expression in M1/M2 mixed phenotype which strengthens the underlying therapeutic efficacy of VA as an immunomodulatory compound. The ability of viscum to manipulate M2 macrophages towards M1 phenotype by increased expression of M1 markers such as CD80, CCR7 and IL-12, and reduced expression of M2 markers such as CD206, CD209 and IL-10, indirectly suggests recruitment of Th1 response and a possibility of relief of tumor immunosuppression, which may represent potential effective anti-tumor therapeutic benefit of VA.

Conclusion

A better understanding of the interplay between myelomonocytic cells and neoplastic cells may confer novel targets for therapeutic intervention and enhancing the anti-tumor response. Balance in monocyte- macrophage function is important for developing immune response which is resistance to pathogens, tissue damage and repair. *Viscum album* is known to improve quality of life of cancer patients and their survival. Our results demonstrate the ability of VA Qu Spez to switch immunoregulatory M2 macrophage cells towards M1, thus indicating the ability of VA to drive Th1 response. This study provides another new angle to understand VA-associated immunomodulation which could be critical in understanding their role as complimentary therapy in cancer.

Methods

VA preparation

VA Qu Spez was a kind gift from Weleda AG (Arlesheim, Switzerland). This is the extract of *Viscum album* growing on different oak trees (Quercus). The VA preparations are therapeutic preparations that are free from endotoxins. VA preparations are formulated in sodium chloride (NaCl 0.9%) isotonic solution as 5 mg/ml vials. VA preparations are prepared by

standardizing the levels of Mistletoe lectins and viscotoxins. VA Qu Spez is a fermented preparation, composed of 785 +/- 10% ng/ml of lectin and 5 +/- 5% µg/ml of viscotoxin.

Isolation of circulating human monocytes, their differentiation to macrophages containing a mixed M1/M2 population

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors purchased from Centre Necker-Cabanel (Etablissement Franc¸ ais du Sang, Paris, France). Circulating monocytes were isolated using CD14 beads (MiltenyiBiotec, Paris,France), and subsequently cultured for 5 days in medium (RPMI 1640, GIBCO/Invitrogen) with 10% FCS, 50 U/ml penicillin, 50 µg/ml streptomycin, and either with 1000 IU/10^6 cells recombinant human GM-CSF (MiltenyiBiotec) to generate M1 macrophage or 2000 IU/10^6recombinant M-CSF (Immunotools) to generate M2 macrophage. Post 5 days macrophage polarization was obtained by removing the culture media and culturing cells for additional 48 hours in the similar medium condition with 200 ng ml^{-1} LPS (from *Escherichia coli*, sigma-Aldrich) plus 40 ng ml^{-1} IFN-γ (Immunotools) for M1 polarization or 500 IU ml^{-1} IL-4 (MiltenyiBiotec) plus 200 ng ml^{-1} IL-13 (Immunotools) for M2 polarization and at the same time cells are co-treated with VA Qu Spez for the subsequent hours.

Isolation of circulating human monocytes and their polarization to distinct M1 and M2 macrophages

Human peripheral blood mononuclear cells were isolated using density gradient centrifugation. Monocytes were isolated using anti-CD14 microbeads (MiltenyiBiotec, Paris, France). Macrophages were obtained by culturing monocytes for 6 days in RPMI 1640 supplemented with 20% FCS plus 50 U/ml penicillin and 50 µg/ml streptomycin and 100 ng ml^{-1}macrophage colony-stimulating factor at a density of 1.5 $\times 10^5$ per cm^2. Macrophage polarization was obtained by removing the culture media and culturing cells for an additional 72 hours in RPMI 1640 supplemented with 5% FCS and 100 ng ml^{-1} LPS plus 20 ng ml^{-1} IFN-γ (for M1 polarization) or 20 ng ml^{-1} IL4 plus 20 ng ml^{-1} IL-13 (for M2 polarization). Post 72 hours M1 and M2 macrophages were treated with VA Qu Spez for 48 hours.

Viscum album treatment

In case of the first protocol, where we obtained a mixed population of M1 and M2, cells were treated with VA Qu Spez for 48 hours at two different concentrations, i.e., 15 µg/ml and 20 µg/ml or they were untreated (control cells). The phenotype of cells was analyzed by flow cytometry. When we obtained distinct M1 and M2 phenotypic cells, they were either

untreated (control cells) or treated with VA Qu Spez at much lower concentration, i.e., 0.5 µg/ml and 1 µg/ml respectively as these concentrations were optimum to the cells. Further cell phenotype was analyzed by flow cytometry.

Flow cytometry

To investigate the effect of VA on macrophages, 0.5×10^6 macrophages were either untreated or treated with VA preparations for 48 hrs. To analyse M1 macrophage phenotype we used the following fluorochrome-conjugated antibodies such as: PE-conjugated-CD80 (BD Biosciences) and APC-conjugated-CCR7 (eBioscience) and to analyze M2 macrophage phenotype, we used PE-conjugated-CD206 (BD Biosciences) and APC-conjugated-CD209 (BD Biosciences).

For determining intracellular M1/M2 expression we used APC-conjugated IL-12 (eBioscience) and PE-conjugated IL-10 (BD Biosciences).

For surface staining cells were suspended in 10% FCS/PBS and antibodies against surface molecules were added at pre-determined concentration and incubated at 4° C for 30 min. BD Fix buffer was used to fix the cells and washed before analysis. The data are presented as % positive cells for indicated molecules or mean fluorescence intensities (MFI) of their expression.

Macrophage cells were stimulated with Phytohaemagglutinin (PHA)-L (10 µg ml^{-1}, Sigma-Aldrich) at 37°C for 18 hours and for extra 2 hours with golgistop (BD Biosciences). Cells were fixed and permeabilized using Foxp3 Fixation/Permeabilization kit (eBioscience) and incubated at 4°C with fluorescence-conjugated mAbs. Cells were acquired on LSRII, 5000 events were recorded and analyzed for each sample. Data was analyzed by BD FACSDIVA software (BD Biosciences, France).

Statistical analysis

Levels of significance for comparison between samples were determined by One way ANOVA Tukey's Multiple Comparison Test. Values of $P < .05$ were considered statistically correlated ($*P < 0.05$, $*** p < 0.0001$). All statistical analyses were performed using Prism 5 software (GraphPad Software, Inc, La Jolla, Calif).

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Figure Legends

Figure.1. VA Qu Spez favours M1 MΦ by reducing M2 surface marker CD206 in a mixed M1/M2 MΦ population of cells. The bar graphs show expression of several MΦ surface markers on M1 and M2 mixed human macrophage cell population treated or untreated with VA Qu Spez in a dose dependent manner. In all the panels X axis indicates concentration of VA Qu Spez, i.e., 15 µg/ml and 20 µg/ml respectively. Untreated macrophage cells are considered as control cells. The expressions of all the markers are analyzed by flow cytometry (BD LSRII). A) % positive cells expressing CD80: M1marker. B) Mean fluorescence intensity (MFI) of CD80 expressing macrophage cells. C) Mean fluorescence intensity (MFI) of the M2 surface marker CD206. Data are mean ± SD, representative of 3 independent experiments done and the statistical significance (*, p <0.05) as analyzed by One-way ANOVA Tukey's multiple comparison Test.

Figure.2. VA Qu Spez efficiently drives M1 phenotypic cells in a M1/M2 MΦ mixed population by significantly upregulating M1 MΦ surface markers such as CD80 and CCR7. Human monocyte- derived macrophage cells were treated either with LPS and IFN-γ for M1 polarization or with IL-4 and IL-13 for M2 polarization. These M1/M2 mixed phenotypic macrophage cells were treated with VA Qu Spez for 48 hours and analysed for surface marker expression by flow cytometry. A) Mean fluorescence intensity (MFI) of CD206. B) % of macrophage cells positive for CD80. C) Mean fluorescence intensity (MFI) of CD80. D) % positive cells expressing CCR7 (M1 phenotype marker). E) Mean fluorescence intensity (MFI) of CCR7. Data are presented as mean ± SEM from minimum 3 independent donors. Statistical significance (*, p< 0.05) as analyzed by One-way ANOVA Tukey's multiple comparison Test.

Figure.3. VA Qu Spez promotes M1 cells in a M1/M2 MΦ mixed population by significant induction of IL-12. Flow cytometric analysis of intracellular IL-12 and IL-10 in M1/M2 macrophage population after 5 days of culture for macrophage polarization. Cells were stimulated with PHA for 18 hours and extra 2 hours of golgi stop stimulation. A-D) Percentage of IL-12 and IL-10 cells (mean ± SEMs, n=3 donors) in M1/M2 polarized macrophages. *, p< 0.05, ***, p< 0.0001, One-way ANOVA Tukey's multiple comparison Test.

Figure.4. VA Qu Spez slightly induces M1 marker CCR7 expression in M1 MΦ cells. 6 days old monocyte-derived macrophages were cultured for 72 hours with LPS and IFN-γ to

obtain M1 macrophages. These M1 cells were treated with or without VA Qu Spez for additional 48 hours and then analysed for surface marker expression by flow cytometry. A) Validation of M1 macrophage polarization indicated by significant % positive expression of M1 surface markers such as CD80 and CCR7. B) Percentage of cells positive for CD80. C) Mean fluorescence intensity (MFI) of CD80 D) % positive cells expressing CCR7. E) Mean fluorescence intensity (MFI) of CCR7 expression. Results are presented as mean ± SEM from minimum 3 healthy donors.

Figure.5. Indication of M2 MΦ switch to M1 MΦ by VA Qu Spez by significant induction of M1 marker CD80. Post 6 days monocyte-derived macrophages were cultured for 72 hours with IL-13 and IL-4 to obtain M2 macrophages. M2 cells were treated with or without VA Qu Spez for 48 hours. The expressions of all the surface markers are analyzed by BD LSRII flow cytometry. A) Conformation of M2 phenotypic expression indicated by significant expression of the mannose-receptor (C-type lectin) CD206 and CD209 (DC-SIGN). B) Percentage of CD206 positive cells. C) MFI of CD206. C) % positive cells expressing CD209. D) MFI OF CD209. E) % positive cells for M1 surface marker CD80. Data from minimum 3 donors is presented (mean ± SEM). *Statistical significance as determined by One-way ANOVA Tukey's multiple comparison Test, where $p< 0.05$.

Figure.6. VA Qu Spez favours M2 MΦ switch to M1 MΦ by reducing IL-10 expression in M2 MΦ population. Flow cytometric analysis of intracellular IL-12 and IL-10 in the M1 and M2 macrophage cells after 9 days culture either in presence of LPS and IFN-γ for M1 macrophage polarization or in the presence of IL-13 and IL-4 for M2 macrophage polarization. In all the panels X axis indicates concentration of VA Qu Spez, i.e., 0.5 µg/ml and 1 µg/ml respectively. These were the optimum concentration to be tolerated by the distinct M1 and M2 macrophage cells in this particular culture condition. Results are representative of minimum 3 healthy donors. A) % positive cells for IL-12 in M1 macrophage cells. B) Percentage of IL-10 cells in M1 macrophage cells. C) Percentage of IL-10 in M2 macrophages. D) % positive cells for IL-12 in M2 macrophage cells. Statistical significance (*, $p< 0.05$) as analyzed by One-way ANOVA Tukey's Multiple comparison Test.

Fig. 2

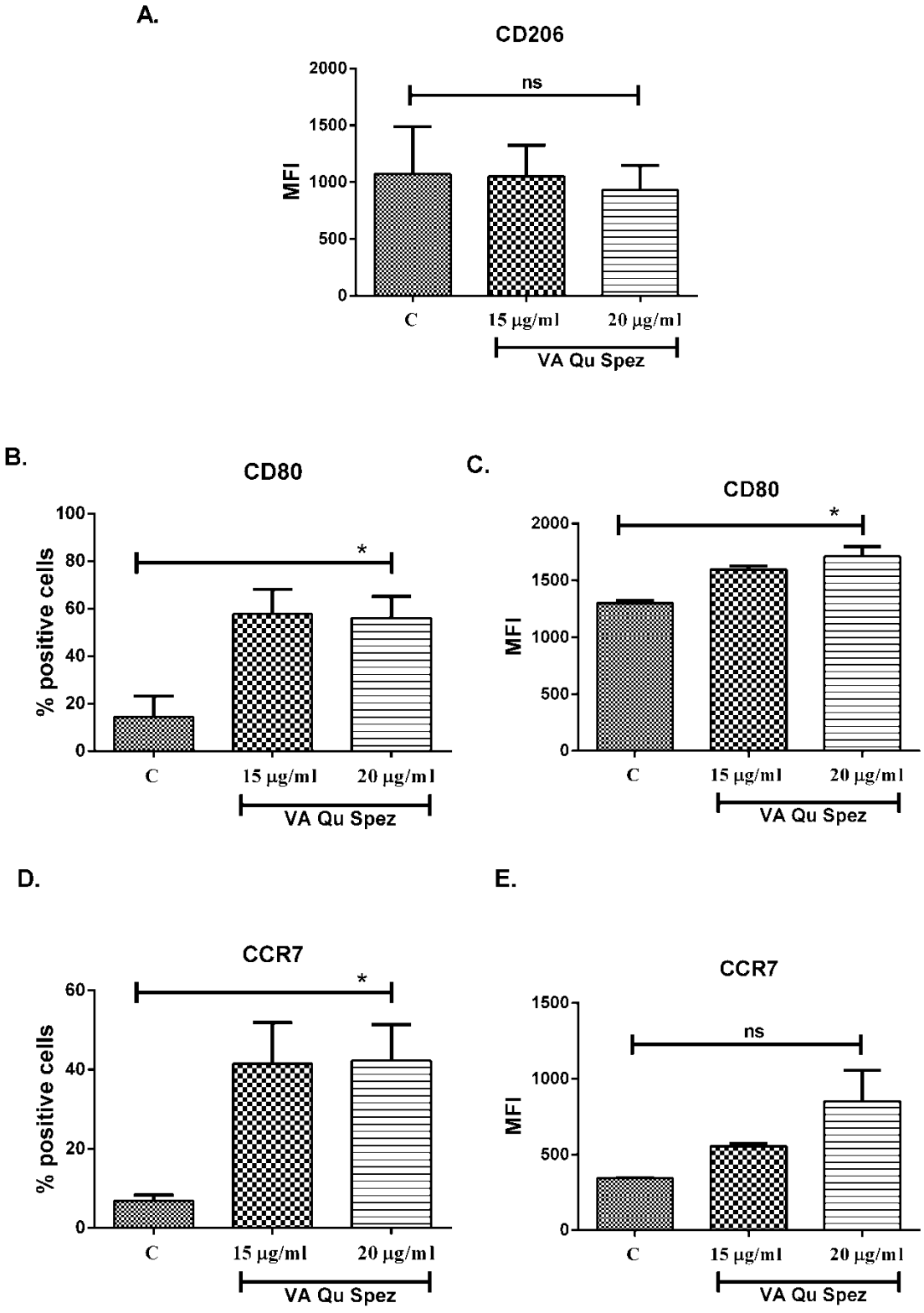
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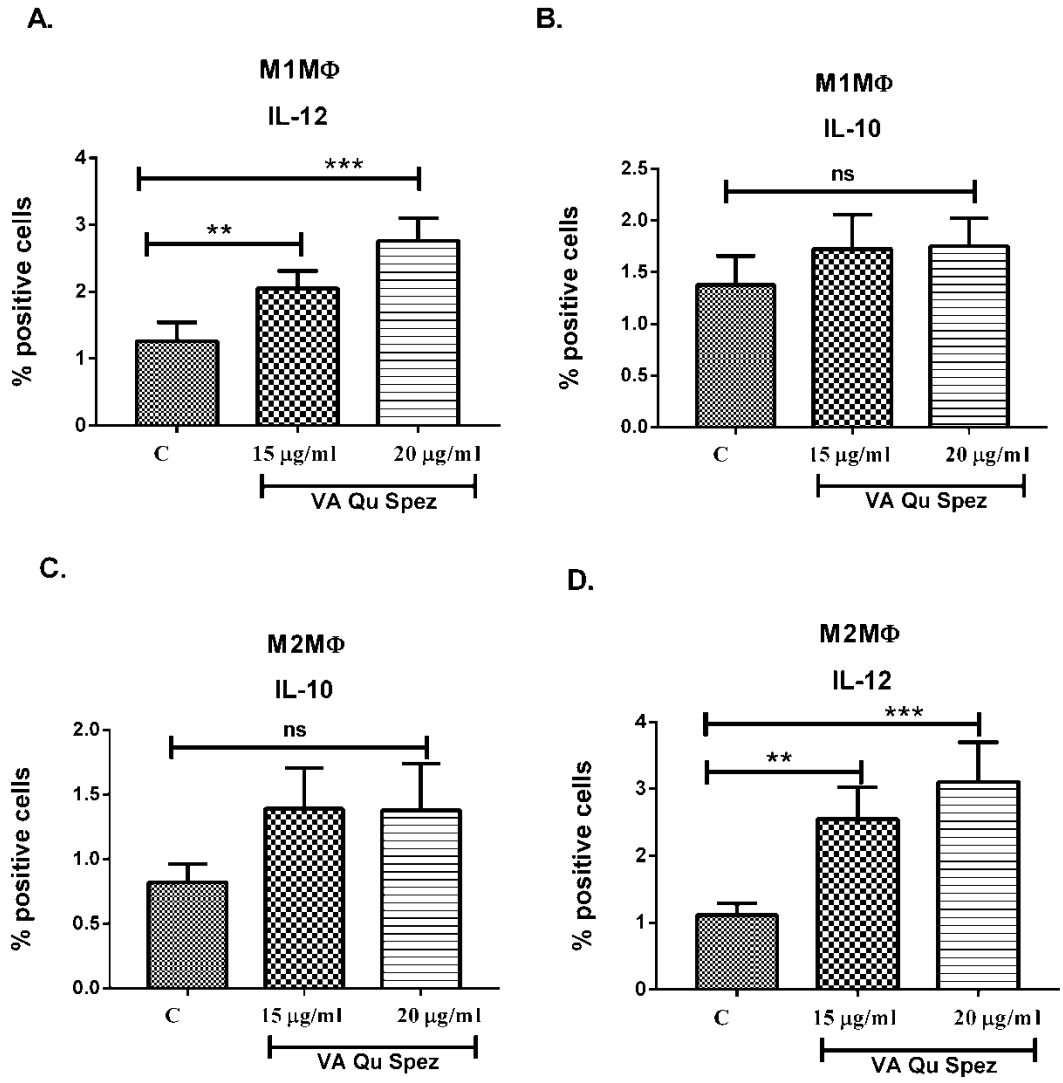
Fig. 3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C.
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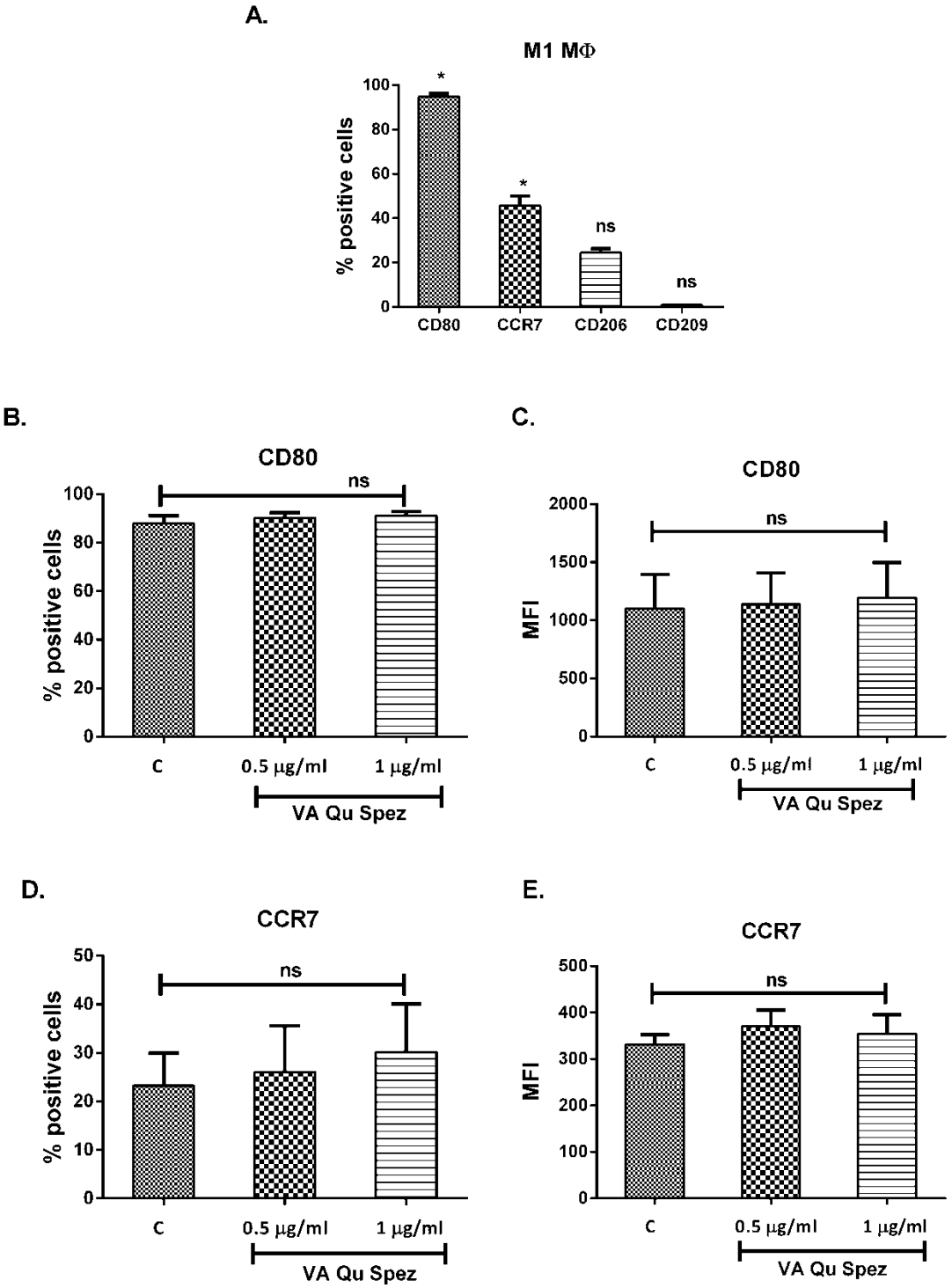
Fig. 4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MFI
2000
1500
1000
500
0
D.
CCR7
ns
% positive cells
50
40
30
20
10
0
E.
CCR7
ns
MFI
500
400
300
200
100
0

Fig. 5

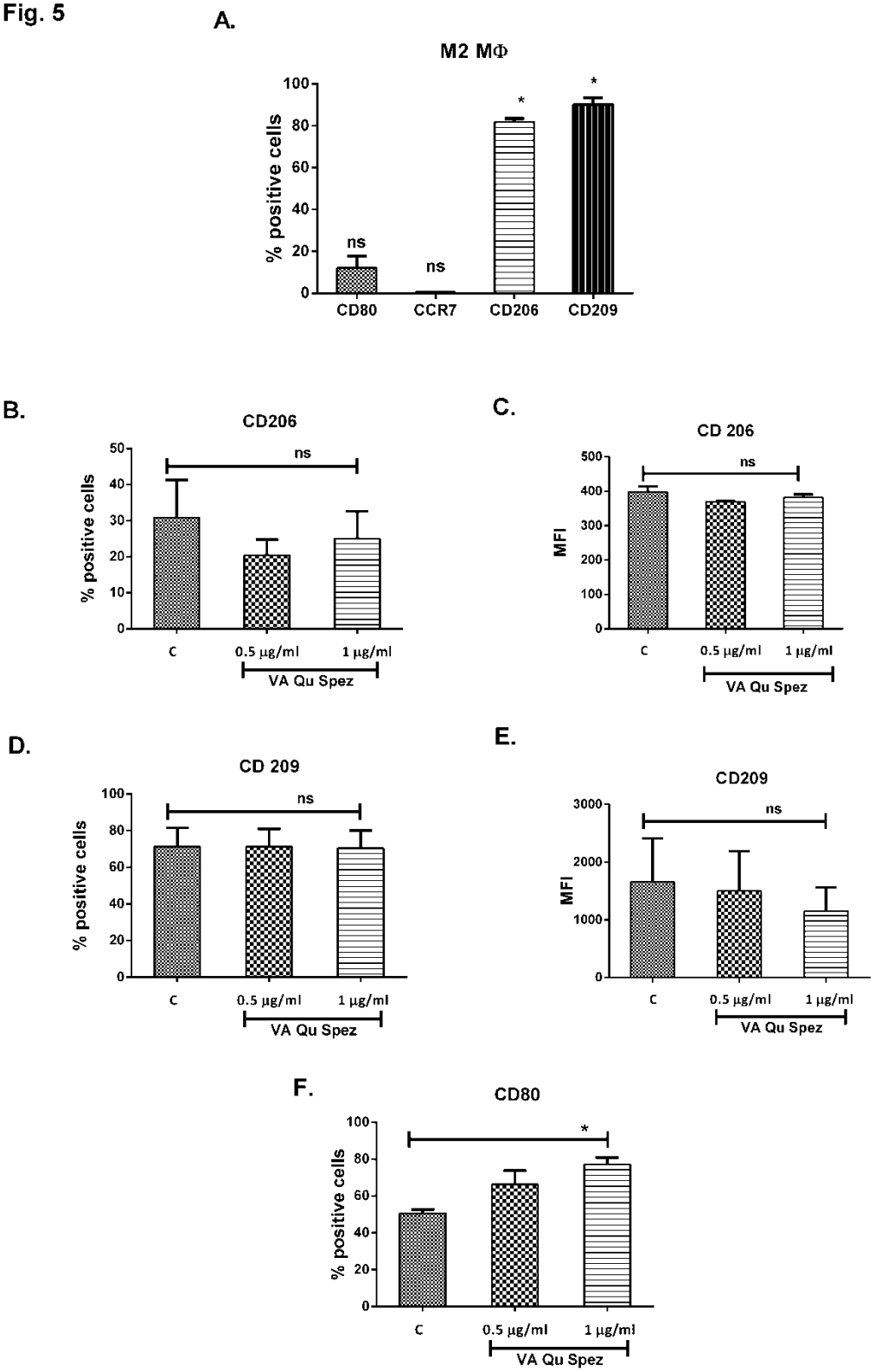
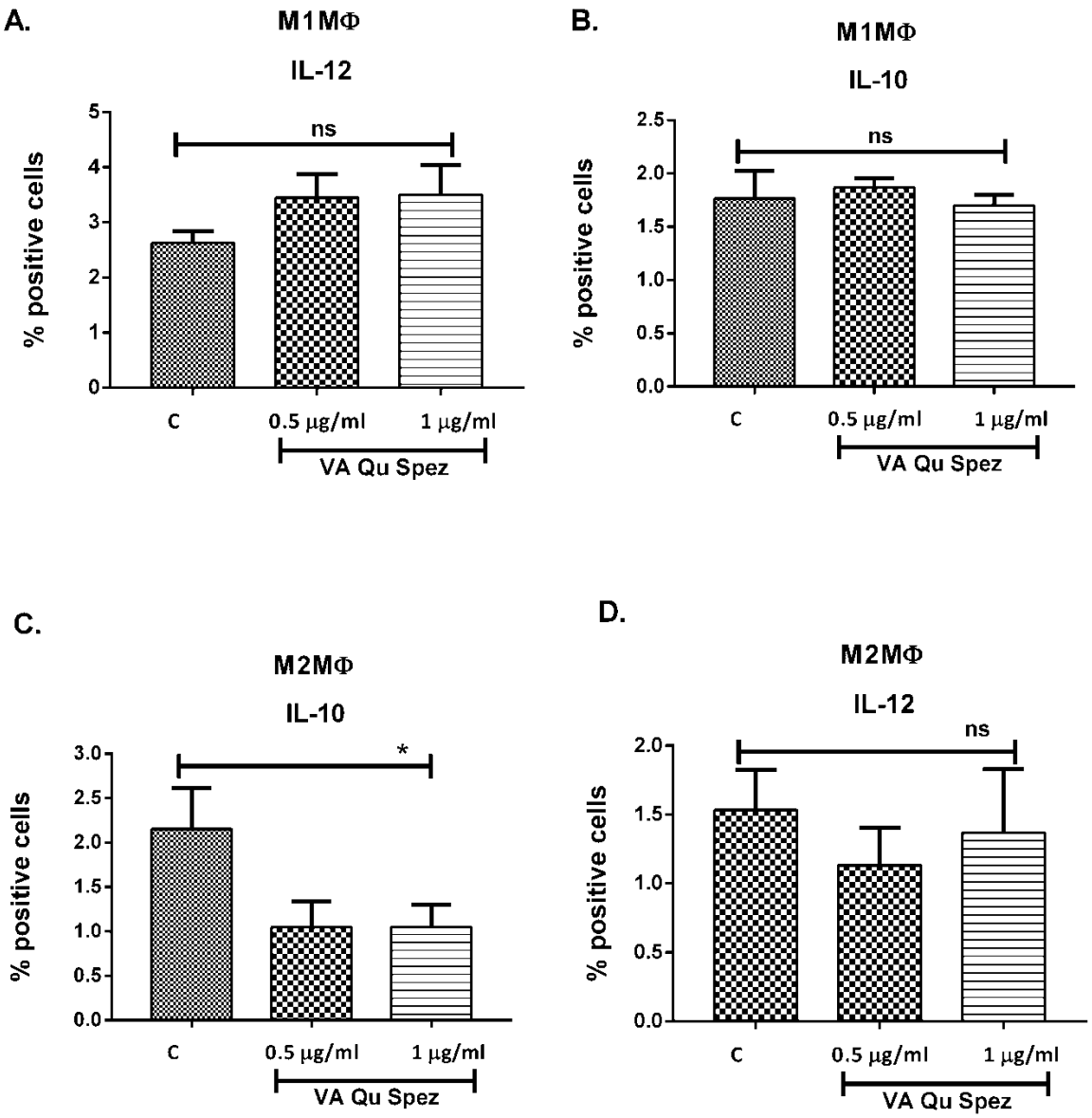

A.
M2 MΦ
% positive cells
100
80
60
40
20
0
ns
ns
*
*
CD80
CCR7
CD206
CD209
B.
CD206
% positive cells
50
40
30
20
10
0
ns
C
0.5 µg/ml
1 µg/ml
VA Qu Spez
C.
CD 206
MFI
500
400
300
200
100
0
ns
C
0.5 µg/ml
1 µg/ml
VA Qu Spez
D.
CD 209
% positive cells
100
80
60
40
20
0
ns
C
0.5 µg/ml
1 µg/ml
VA Qu Spez
E.
CD209
MFI
3000
2000
1000
0
ns
C
0.5 µg/ml
1 µg/ml
VA Qu Spez
F.
CD80
% positive cells
100
80
60
40
20
0
*
C
0.5 µg/ml
1 µg/ml
VA Qu Spez

Fig. 6


A.
M1MΦ
IL-12
% positive cells
ns
C
0.5 µg/ml
1 µg/ml
VA Qu Spez
B.
M1MΦ
IL-10
% positive cells
ns
C
0.5 µg/ml
1 µg/ml
VA Qu Spez
C.
M2MΦ
IL-10
% positive cells
*
C
0.5 µg/ml
1 µg/ml
VA Qu Spez
D.
M2MΦ
IL-12
% positive cells
ns
C
0.5 µg/ml
1 µg/ml
VA Qu Spez

DISCUSSION
&
PERSPECTIVES

The immune system can discriminate between a range of stimuli, in one hand it allows some to provoke immune responses, which leads to immunity, and on the other hand it can prevent others from doing so, which leads to tolerance. To this end, the immune system is highly organized and orchestrated at molecular, cellular and systemic levels. In the context of tumour immunology, tumour immunity or immune tolerance to tumour means the success or failure of the immune system to eliminate a tumour. When the immune tolerance is interrupted, an exacerbated activity of the immune system towards self-tissues leads to autoimmune disease characterized by inflammation. Inflammation is a physiopathological symptom of infection, autoimmunity or cancer. Thus, dysfunctional immune system serves as an ideal target for several therapeutic interventions. Therapeutic intervention using natural compounds has enormously contributed to the field of complementary and alternative medicines (CAM). A prominent group of effective cancer chemo preventive drugs has been derived from natural products having low toxicity while possessing apparent benefit in the disease process. Our research group is extremely interested in elucidating the immunomodulatory effects of *Viscum album,* a heterogeneous plant preparation, extensively used in the complementary and alternative medicine for cancer for several years. Several preclinical and clinical data suggest that *Viscum album* improves the survival and quality of life of cancer patients. In addition, VA preparations have been implicated as conventional phytotherapeutics in the treatment of several conditions associated with allergic reactions, nervous system abnormalities and immune-inflammatory diseases. Long-term side effects of nonsteroidal anti-inflammatory drugs (NSAID) in various pathological conditions and the increasing body of evidence for anti-inflammatory activity of plant-derived molecules together encourage the conception of phytotherapeutics as potent alternatives to classical anti-inflammatory drugs.

Despite the use of viscum in complementary medicine and their therapeutic benefit, being observed for several decades, the underlying molecular mechanisms have not been clearly demonstrated. Therefore, my study is focused towards understanding the molecular mechanisms of these therapeutic preparations, in the context of inflammation and majorly immunomodulation.

Viscum album-Mediated COX-2 Inhibition Implicates Destabilization of COX-2 mRNA

Owing to the intricate association of inflammation and cancer and in view of the fact that several anti-tumor phytotherapeutics also exert a potent anti-inflammatory effect, our group have recently demonstrated that, VA Qu Spez exerts a potent anti-inflammatory effect by selectively downregulating the COX-2-mediated cytokine-induced secretion of prostaglandin

E2 (PGE2), one of the important molecular signatures of inflammatory reactions. Therefore, we hypothesized that VA induces destabilisation of COX-2 mRNA, thereby depleting the available functional COX-2 mRNA for the protein synthesis and for the subsequent secretion of PGE2.

Clinical demonstration of severe side effects due to the failure of the classical COX-2 inhibitors to discriminate between an aberrant pathological versus homeostatic functional activation state, raised the concern that direct COX-2 enzymatic inhibition might not sufficiently represent an appropriate clinical strategy to target COX-2. COX-2 is an early response gene, similar to the genes encoded for cytokines, chemokines and proto-oncogenes, they can be regulated under different levels of expression and modulation, ranging from direct transcriptional effects to post-transcriptional and post-translational levels and also indirectly by various transcription factors that mediate the stability. Such multiple levels of modulation of COX-2 expression imply the existence of several mechanisms, which may be targeted to finely modulate COX-2 functions. Several phytotherapeutics have been shown to exert modulatory effect on COX-2 at various levels of its molecular regulation and therefore have been considered as an effective alternative strategy to control the pathogenic expression of COX-2.

Thus, I analyzed the protein stability of COX-2 in the presence of VA by cyclohexamide pulse chase experiments. Flow cytometric analysis of COX-2 expression after 90 minutes of blocking the protein synthesis with cyclohexamide showed that, there is no significant difference in the COX-2 degradation profile of cells simulated with IL-1β with or without treatment with VA. Western blot analysis of COX-2 protein after 5 and 11 hours of cyclohexamide blockade showed no significant difference in the degradation pattern of COX-2 in cytokine stimulated cells with or without VA treatment. Similar results at different time points were observed. Therefore, it is clear that COX-2 protein degradation is not affected by VA. Further, reduced level of COX-2 expression at 0 hour in this experiment also suggests that, there may be modulation by VA of the COX-2 expression before the addition of inhibitor of protein synthesis. Inhibition of COX-2 protein expression by VA without modulating its stability strongly indicates that, there is a possible modulation by VA at an early stage than when the proteins were expressed. However VA did not modulate COX-2 mRNA expression and therefore, I analyzed the mRNA stability of COX-2 by actinomycin D pulse chase experiment. mRNA degradation profile of COX-2 obtained by analyzing the COX-2 mRNA at different time intervals after blocking the transcription using actinomycin D showed that the rate of degradation of COX-2 mRNA is higher in cells treated with VA compared to those treated with cytokine alone. This reduction in the mRNA half-life of COX-2 in the cells

treated with VA suggests that, VA induces destabilization of COX-2 mRNA, thereby diminishing the available functional mRNA for the protein synthesis. Although this study postulates destabilization of COX-2 mRNA by VA preparations as a possible mechanism for VA-mediated COX-2 inhibition, further molecular dissection is necessary in order to clearly understand the regulatory events of COX-2 regulation, contributing factors and their modulation by VA preparations.

Differential effect of various preparations of *Viscum album* on maturation and activation of human dendritic cells and T cell response

In addition to the anti-inflammatory and cytotoxic properties, VA preparations have immunostimulatory effects. However, to mount an effective anti-tumoral immune response, an induced expression of co-stimulatory molecules on the DCs, the sentinels of the immune system, which bridges the innate immune system with adaptive immune system, accompanied by an enhanced secretion of pro-inflammatory cytokines that culminates in T cell proliferation is required. There are several reports, suggesting that in breast cancer patients DCs found to exert deficiencies in expressing several co-stimulatory molecules and impaired generation of inflammatory cytokines. Our group had demonstrated that mistletoe extracts induce maturation and activation of DCs accompanied by the induction of inflammatory cytokines and stimulation of tumor-specific T cells. It is a well-documented fact that mistletoe extracts are heterogeneous preparations and thus exert different response. The difference in the biological activities of different *Viscum album* majorly depends on the host trees or the nutritional source, the time they are harvested or seasonal variation, and the method of extraction. Therefore, I was interested to perform a comparative study of different preparations of *Viscum album* which could be an important contribution to the field to understand the underlying mechanism of action of each VA preparations associated with its anti-tumor effect. In my study, I included five different preparations of VA - VA Qu Spez, VA Qu Frf, VA M Spez, VAP and VAA respectively. I demonstrated the differential effect of these preparations on maturation and activation of dendritic cells (DCs) which in turn may manifest anti-tumoral immune response. We found among all five preparations, VA Qu Spez significantly induces DC activation and secretion of pro-inflammatory cytokines such as IL-6, Il-8 and TNF-α, enhancing IFN-γ production hence promoting Th1 immune response. Flow cytometric data revealed that VA Qu Frf, VA M Spez, VAP and VAA were not able to enhance the expression of antigen presenting molecule HLADR and co-stimulatory molecules such as CD80, CD86, CD83, CD40. But interestingly, VA Qu Spez was able to enhance the expression of the co-stimulatory as well as the antigen presenting molecules. Further, I analyzed the differential effect of five VA preparations on secretory cytokines such as IL-6,

IL-8, IL-10 and TNF-α. Results showed apart from VA Qu Spez, VA Qu Frf and VA M Spez showed moderate enhancement of these pro-inflammatory cytokines, but VAP and VAA were unable to modulate their secretion level not even at considerable level. VA Qu Spez is efficiently capable of enhancing IL-6, IL-8 and TNF-α, without having any effect on immune-suppressive cytokine IL-10. One of the key functions of APC is to promote polarized T cell response. Thus, next explored the effect of VA Qu Spez on T cell responses. Five preparations of VA primed DCs were co-cultured with allogenic total $CD4^+$T cells at a ratio of 1:10 and Th1, Th2,Th17 and Treg response was measured by flowcytometric analysis of intracellular IL-17 (Th17), IFN-γ (Th1), IL-4 (Th2), FOXP3 (Treg). Previous data suggested that VA Qu Spez is capable of activating DCs differentially, but these activated DCs were unable to promote any of the T cell response analyzed by flow cytometry. Further, I sustained the observation by analyzing the quantity of IFN-γ, IL-4, IL-13 and Il-17 in culture supernatants by ELISA. Data revealed that VA Qu Spez significantly stimulates IFN-γ secretion without having any effect on modulation of IL-4, IL-13, and IL-17, suggesting VA Qu Spez favours Th1 response.

Cytotoxicity of mistletoe is majorly attributed to its lectins. As mistletoe lectins bind to sugar residues, it is meaningful to speculate that glycoproteins from serum competes with cell surface glycoproteins for binding of lectin, thus abrogating the amount of lectins engulfed by cells. Cytotoxic glycoproteins, the lectins, are one of the active components of mistletoe extracts which is responsible for stimulating effector cells of the innate and adaptive immune system such as dendritic cells, macrophages, natural killer cells, and B and T lymphocytes, that might be one of the mechanisms responsible for the anti-tumoral effect of mistletoe extracts. Several studies clearly demonstrated lectin internalization is required for ML-I mediated apoptosis independent of surface receptor- mediated pathway. Among all five preparations, VA Qu Spez contains the second highest amount of lectin, i.e., 785 +/- 10% ng/ml, this could one of the possible reasons to account for it to be the most effective preparation in terms of DC activation and conferring T cell response.

For therapeutic intervention at least three approaches can be considered to induce tumor rejection by cytotoxic T lymphocytes (CTLs). First is encouraging antigen presenting ability of DCs, second is promoting protective T cell response and last but not the least defeating immunosuppressants in tumor vicinity. Several DC-based cancer immunotherapy has been established with the aim of enhancing DC maturation with elevated expression of maturation-molecules, high migration capacity, enhancing CTLs. Tumor apoptosis can be achieved by Idiotype specific $CD4^+$ Th1 cell by FasLFas interaction directly and indirectly by IFN-γ production which can regress tumor. Our data demonstrates that VA Qu Spez induces IFN-γ

production thus favouring Th1 type immune response which indirectly could be one of the mechanisms strengthening the beneficial effect of VA preparations as complimentary therapy in cancer.

***Viscum album* promotes anti-tumor response by modulating M1/M2 macrophage polarization switch**

Mechanisms of immune surveillance are able to control the growth of new tumors or affect the progression of existing tumors is well known. Immune dysregulation and immune suppression in cancer patients are the composite events which in turn leads to abnormal myelopoesis and recruitment of several immunosuppressive myelomonocytic cells at tumor vicinity. This immune dysfunction even can cause death of tumor-speecific T cells or lymphocyte dysfunction. These immunosuppressive myelomonocytic cells are involved in process of angiogenesis and stroma remodelling needed for tumor progression. In a tumor site, tumor-derived factors are involved in functional differentiation of myelomonocytic cells majorly the macrophages to sustain myelopoiesis. The orchestration of myelomonocytic cell function links inflammation and cancer. Macrophages show considerable functional plasticity and exert an immediate respond to alter according to the change in the microenvironment. Cancer serves a paradigm of macrophage plasticity and diversity. Alberto Mantovani described that, in the tumor site, circulating monocytes are recruited and these cells differentiate into tumor associated macrophages (TAMs). These TAMs play an important role in tumor progression by encouraging inflammation and angiogenesis thus inducing neoplastic growth. Based on Th1 and Th2 polarization, macrophages are classified in two distinct states: the classically activated macrophages or M1 and the alternatively activated macrophages or M2. M1 macrophages recruit Th1 cells thus promoting Th1 response and they confer resistance to intracellular pathogens and tumors leading to tumor-disruptive response. In contrast, as M2 macrophages drive Th2 immune response in turn they actively participate in encapsulation and clearance of parasites hence in tumor progression and tissue remodelling and importantly exert an immune regulatory property. Therefore, in view of the anti-tumoral and immunomodulatory effect of viscum, I was interested to investigate whether VA can modulate macrophage polarization, which in part explains the mechanisms underlying the beneficial effect of VA in cancer therapy. In the present *in vitro* study, I demonstrated that VA Qu Spez drives M2 switch towards M1, in a mixed M1/M2 population as well as in distinct M1 and M2 phenotype. VA- mediated modulation of this switch is demonstrated by induced expression of M1 surface markers such as CD80 and CCR7, but in parallel, reduced expression of M2 surface markers such as CD206 and CD209, with VA treatment. In addition, VA provoked polarization of IL-12^{hi} and IL-10^{lo} characterised M1cells over $IL12^{lo}$

and IL-10^{hi} M2 macrophages, suggesting that VA is able to play M2 to M1 switch. There are several reports suggesting, M2-like pro-tumoral phenotype of TAMs in cancer is reversible. Tumor associated macrophages are the main source of IL-10 which is an imuunomodulating molecule which can turn down innate and adaptive immune response and they are responsible to maintain M2 state of macrophages. Strategies to overcome tumor escape mechanisms by changing the immunosuppressive situation are the key to effective immunotherapy. My results show that VA down modulates IL-10 expression in M2 macrophages will probably be one of the reasons for VA to exert immunomodulatory effect. IL-12 links innate and adaptive immunity by promoting cytotoxic T lymphocytes (CTL). Reduced production of CTL and abrogation of tumor rejection was observed in IL-12 knockout mice. In this study we show that VA Qu Spez was able to significantly induce IL-12 expression in M1/M2 mixed phenotype which strengthens the underlying therapeutic efficacy of VA as an immunomodulatory compound. The ability of viscum to manipulate M2 macrophages towards M1 phenotype by increased expression of M1 markers such as CD80, CCR7 and IL-12, and reduced expression of M2 markers such as CD206, CD209 and IL-10, indirectly suggests recruitment of Th1 response, which may represent potential effective anti-tumor therapeutic benefit of VA.

Altogether, the aim of my thesis to understand the anti-inflammatory and immunomodulatory effect of *Viscum album,* I found that VA exerts anti-inflammatory effects by selectively inhibiting COX-2 expression and ensuing PGE2 production, implicating viscum mediated COX-2 mRNA destabilisation. An *in vitro* comparative study involving five different preparations of VA concerning maturation and activation of dendritic cells (DCs) which in turn may manifest anti-tumoral immune response, showed that among all five preparations, VA Qu Spez significantly induces DC activation, secretion of pro-inflammatory cytokines such as IL-6, Il-8 and TNF-α, enhancing IFN-γ production hence promoting Th1 immune response. In view of the key role of myelomonocytic cells that links inflammation and cancer and provides a paradigm for macrophage plasticity and function, my study revealed an interesting effect of VA Qu Spez in switching the M2 macrophages which are known to participate in polarizing Th2 responses, help with parasite clearance, dampen inflammation, promote tissue remodelling and tumor progression and have immunoregulatory functions, towards classically activated M1 macrophages which are part of a polarized Th1 response and mediate resistance to intracellular pathogens and tumors and elicit tissue-disruptive reactions.

Perspectives

Although viscum is known for several years for their traditional and medicinal history and known to exert several therapeutic benefits, an extensive future investigation is required with respect to its mechanism of action at systemic, cellular and molecular level.

Thus, I propose some of the future direction of this study to extend my thesis studies which broadly include:

- **TLR4-mediated activation of immune cells: To explore the downstream cell signalling target of viscum**

- **To decipher the downstream target of *Viscum album* associated with viscum-mediated M2 to M1 polarization switch: better understanding of therapeutic benefit of viscum**

- **To explore the clinical relevance of immunomodulatory effect of *Visum album*: angle of viscum mediated DC activation and macrophage polarization switch**

TLR4-mediated activation of immune cells: To explore the downstream cell signalling target of viscum

The main immunostimulatory component of *Viscum album* preparation, mistletoe lectin, has been recently shown to be a pattern recognition receptor ligand, which binds to an important class of pathogen-sensing receptors. Pattern recognition receptor ligands are key players of cancer immunotherapy, which are responsible for activating dendritic cells, leading to T cell response against cancer cells. Recent study revealed a structural similarity between bacterial origin mistletoe lectin and shiga toxin from *Shigella dysenteriae*, which describes immunogenicity of mistletoe lectin (Maletzki, Linnebacher et al. 2013). Another study suggests Korean mistletoe lectin is a Toll-like receptor (TLR) 4 ligand (Park, Hong et al. 2010). As European mistletoe and Korean mistletoe have 84% of sequence resemblance and similar 3D structures, suggesting European mistletoe presumably is a TLR-4 ligand (Abagyan and Batalov 1997). The TLR family is one of the best identified PRR families responsible for sensing invading pathogens outside of the cells and intracellular endosomes and lysosomes (Akira, Uematsu et al. 2006). TLRs can recognise PAMPs and this can lead to transcriptional upregulation of distinct genes, which depends on the TLRs and the cell types (Maletzki,

Linnebacher et al. 2013). Based on the selection of the distinct adaptor molecules, TLR signalling is divided into two broad pathways, MyD88 and TRIF. TLR4 together with myeloid differentiation factor 2 (MD2) recognize lipopolysaccharide (LPS) on the cell surface. TLR4 triggers both MyD88-dependent and TRIF-dependent signalling. TLR4 is involved in severity of inflammation induced by nonmicrobial agents. MyD88 interacts with IL-1R-associated kinase (IRAK)-4, which activates other IRAK family members such as IRAK-1 and IRAK-2. The IRAKs further dissociate from MyD88 and interact with TNFR-associated factor 6 (TRAF6), which acts as an E3 ubiquitin protein ligase. Downstream of this pathway, TGF-β-activated kinase 1 (TAK1) along with TAK1-binding protein1 (TAB1), TAB2, and TAB3 is activated and phosphorylates IκB kinase (IKK)-β and MAP kinase kinase 6. IKK-α, IKK-β, and NF-κB essential modulator (NEMO), together known as the IKK complex, phosphorylates an NF-κB inhibitory protein IκBa. This phosphorylated IκB undergoes degradation by ubiquitin-proteosome system, which in turn helps NF-κB to translocate to the nucleus and activates the expression of several pro-inflammatory cytokine genes (Maletzki, Linnebacher et al. 2013). TLR signalling induces expression of noncoding RNAs, which can lead to production of microRNAs (Guttman, Amit et al. 2009), (Taganov, Boldin et al. 2006). TLR4 signalling is negatively modulated by miR-21 by the tumor suppressor PDCD4, which is required for NF-κB activation (Sheedy, Palsson-McDermott et al. 2010). In view of mistletoe being a TLR4 ligand and active participation of TLR4 in triggering the intracellular signalling cascades, leading to transcriptional expression of inflammatory mediators that coordinate the elimination of pathogens and infected cells, it would be interesting to unravel the underlying molecular mechanism of viscum which can support its immunomodulatory effect. Though the wide use of viscum as successful adjuvant therapy is known, but their mechanism of action is yet to be explored extensively. Therefore investigating the target of viscum in these downstream signalling pathways and identifying the microRNA target of viscum, which underlies its therapeutic benefit could be an interesting angle to explore to have a better understanding of the specific target of viscum, which in turn might help in the adjuvant therapy of viscum for the cancer patients.

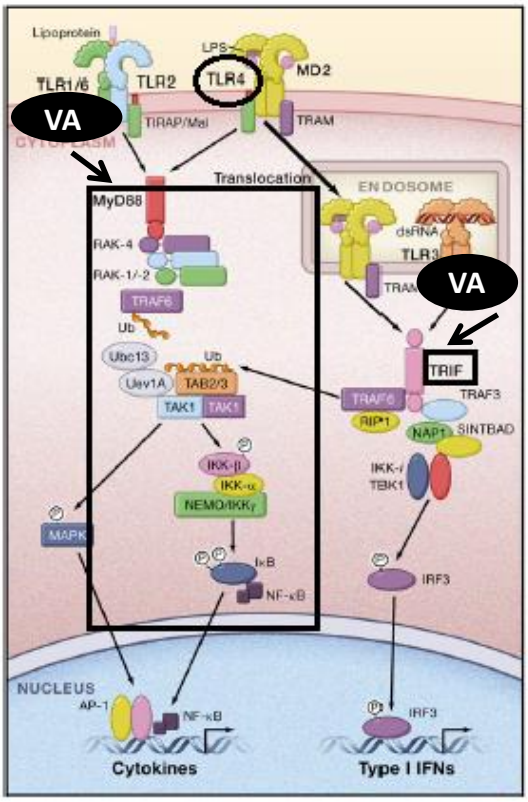


Figure 16: TLR signalling pathways. (Adapted from Shizuo Aira, Cell, 2010)

To decipher the downstream target of *Viscum album* associated with viscum-mediated M2 to M1 polarization switch: better understanding of therapeutic benefit of viscum

LPS and IFN-γ are the major stimuli for M1 polarization, which signal trough TLR4, IFN-α, or IFN-β receptor (IFNAR) and IFN-γ receptor (IFNGR) pathways, inducing activation of the transcription factors such as NF-κB (p65 and p50), AP-1, IRF3 and STAT1, leading to the transcription of M1 genes. STAT6, a master regulator of M2 macrophage polarization, induces the expression of transcription factor PPAR-γ. Histone demethylase JMJD3 regulates transcription of several M2-asscociated genes, such as Arg1, Ym1, and Fizz at an epigenetic level. IL-4 induces upregulation of JMJD3, which in contrast inhibits M1 transcription. JMJD3 regulates M2 polarization by inducing transcription factor IRF4 expression, which is known to be a negative regulator of TLR4 signalling by binding to MyD88. The binding of immune complexes to activatory FcγR on macrophages triggers a tyrosine kinase dependent pathway, which inhibits TLR4 through upregulation of IL-10. Prostaglandin E2 in produced when the inhibitory receptor FcγRIIb on macrophages is ligated, which inhibits TLR4 triggered inflammatory cytokines expression (Biswas and Mantovani 2010). Our results demonstrated *Viscum album* directs the M2 polarization switch towards M1; suggesting

viscum is capable of promoting Th1 response efficiently. Thus, it would be of great interest to dissect the molecular mechanism of action of viscum on this macrophage switch axis. As I described earlier that, there are reports suggesting viscum indeed is a TLR4 ligand and considering the fact of the negative regulation of TLR4 signalling by several M2 specific genes, it would be enthralling to investigate the direct inhibitory effect of viscum on any of these M2 associated downstream signalling cascade.

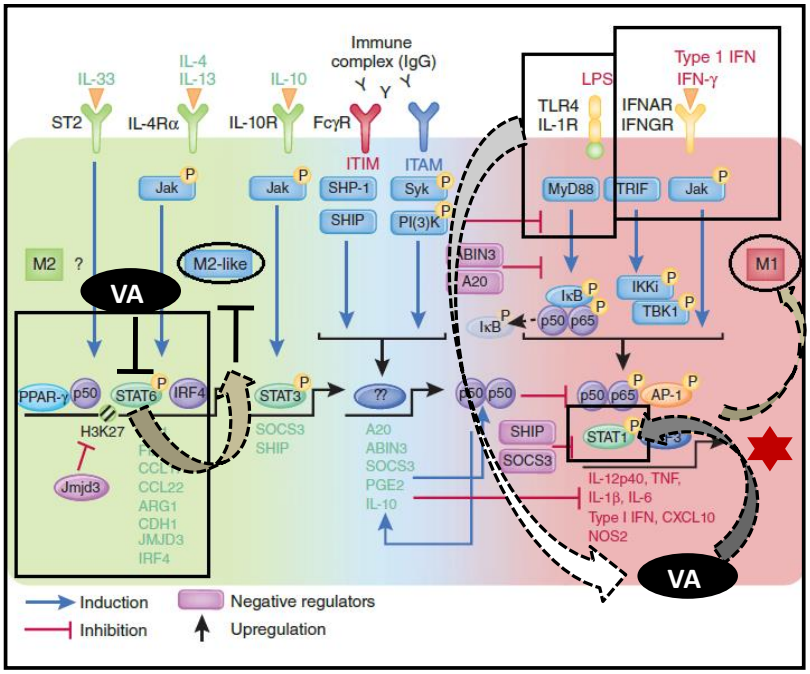


Figure 17: Molecular pathways of macrophage polarization. (Adapted from Subhra K Biswas, nature Immunology, 2010)

To explore the clinical relevance of immunomodulatory effect of *Visum album*: angle of viscum mediated DC activation and macrophage polarization switch

Results presented in my thesis reveal mechanism of action of *Viscum album* in an *in vitro* system. This complete study can be extended to validate the similar observations in cancer patients following viscum therapy and other experimental models of cancer which in turn can strengthen the study.

We are very interested in generating ideas and developing possible preclinical models in collaboration with the scientists in Arlesheim to investigate the effect of viscum treatment on cancer-related fatigue, especially determining the mode of action.

Cancer fatigue is one of the main symptoms that significantly affect the quality of life of patients, which on the other hand is beneficially affected by complementary viscum treatment. Therefore, it is interesting to explore the underlying therapeutic benefit of viscum in the

context of cancer related fatigue considering better understanding of their effective role as an anti-tumor, anti-inflammatory, anti-angiogenic and importantly immunomodulatory compound.

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ANNEXES

List of Publications

1. **Saha C**, Hegde P, Friboulet A, Bayry J, Kaveri SV. Viscum album-mediated COX-2 inhibition implicates destabilization of COX-2 mRNA. *PLoS One* 2015,**10**:e0114965.

2. Stephen-Victor E, **Saha C**, Sharma M, Holla S, Balaji KN, Kaveri SV*, et al.* Inhibition of programmed death 1 ligand 1 on dendritic cells enhances Mycobacterium-mediated interferon gamma (IFN-gamma) production without modulating the frequencies of IFN-gamma-producing CD4+ T cells. *J Infect Dis* 2015,**211**:1027-1029.

3. Elluru SR, **Saha C**, Hegde P, Friboulet A**,** Bayry J and Kaveri SV. 2015**.** Dissecting the anti-inflammatory effects of Viscum album: Inhibition of cytokine-induced expression of cyclooxygenase-2 and secretion of prostaglandin E2. In Mistletoe: From Mythology to Evidence-Based-Medicine (Edited by Zaenker KS, and Kaveri SV). Translational Research in Biomedicine (Chan SHH, Series Editor) vol 4. Karger Publisher, Basel. Page: 67-73.

4. Kaveri SV, Lecerf M, **Saha C**, Kazatchkine MD, Lacroix-Desmazes S, Bayry J. Intravenous immunoglobulin and immune response. *Clin Exp Immunol* 2014,**178 Suppl 1**:94-96.

5. Sharma M, Schoindre Y, Hegde P, **Saha C**, Maddur MS, Stephen-Victor E*, et al.* Intravenous immunoglobulin-induced IL-33 is insufficient to mediate basophil expansion in autoimmune patients. *Sci Rep* 2014,**4**:5672.

6. Sharma M, **Saha C**, Schoindre Y, Gilardin L, Benveniste O, Kaveri SV*, et al.* Interferon-alpha inhibition by intravenous immunoglobulin is independent of modulation of the plasmacytoid dendritic cell population in the circulation: *Arthritis Rheumatol* 2014,**66**:2308-2309.

7. Othy S, Topcu S, **Saha C**, Kothapalli P, Lacroix-Desmazes S, Kasermann F*, et al.* Sialylation may be dispensable for reciprocal modulation of helper T cells by intravenous immunoglobulin. *Eur J Immunol* 2014,**44**:2059-2063..

8. **Saha C**, Friboulet A, Bayry J, Kaveri SV. Differential effect of *Viscum album* preparations on maturation and activation of human dendritic cells and T cell response. (Manuscript No. BBRC-15-5516)

9. **Saha C**, Friboulet A, Bayry J, Kaveri SV. *Viscum album* promotes anti-tumor response by modulating M1/M2 macrophage polarization switch. (Under communication)

Correspondence

Inhibition of Programmed Death 1 Ligand 1 on Dendritic Cells Enhances Mycobacterium-Mediated Interferon γ (IFN-γ) Production Without Modulating the Frequencies of IFN-γ–Producing $CD4^+$ T Cells

To the Editor—Mycobacterium tuberculosis, the causative agent of tuberculosis, uses several strategies to evade the immune system, which include inhibition of phagosomal maturation and antigen presentation, blockade of apoptosis and autophagy of infected cells, suppression of T-helper type 1 (Th1) and interferon γ (IFN-γ) responses, and expansion of $CD4^+CD25^+FoxP3^+$ regulatory T cells (Tregs) [1–3]. Recently Singh et al reported that M. tuberculosis exploits the programmed death 1 (PD-1) pathway to inhibit IFN-γ responses [4]. Conversely, blockade of the PD-1 pathway either by blocking PD-1 on $CD3^+$ T cells or blocking PD-1 ligand 1 (PD-L1) on monocytes in vitro rescued IFN-γ–producing T cells from undergoing apoptosis. However, 2 issues remain unanswered: (1) the specific role of PD-L1 on $CD4^+$ T cells and (2) the contribution of PD-L1 on dendritic cells (DCs), the professional antigen-presenting cells, in polarizing Mycobacterium-mediated IFN-γ responses from naive $CD4^+$ T cells.

Human $CD4^+$ T cells, when activated, were reported to express PD-L1 [5]. Therefore, it is likely that interaction of PD-L1–expressing $CD4^+$ T cells with PD-1–positive T cells might modulate IFN-γ responses. We found that Mycobacterium induced only a marginal increase in PD-L1 expression on $CD4^+$ T cells (Figure 1A). Our results thus indicate that the relatively high expression of PD-L1 on $CD3^+$ T cells (up to 25%) observed by Singh et al [4] upon stimulation with mycobacterial antigens might reflect modulation of PD-L1 expression on $CD8^+$ T cells, rather than $CD4^+$ T cells. PD-L2 expression, however, remained negative on these activated $CD4^+$ T cells (data not shown). In accordance with data on low-level expression of PD-L1 on $CD4^+$ T cells, blockade of this molecule by using monoclonal antibodies (mAbs) did not significantly modulate either the frequency of IFN-γ^+CD4^+ T cells (Figure 1B and 1C) or the quantities of IFN-γ secreted from these cells (Figure 1D). Thus, our results suggest that PD-L1 on $CD4^+$ T cells plays only a marginal role in mediating impaired IFN-γ responses by Mycobacterium.

Dendritic cells (DCs) are sentinels of the immune system that orchestrate primary immune responses to Mycobacterium by polarizing distinct $CD4^+$ T-cell responses from naive T cells [1]. Therefore, we next examined the role of PD-L1 on DCs in regulating IFN-γ polarizing responses from naive $CD4^+$ T cells. DCs were generated from circulating monocytes as previously described [6]. Similar to the results obtained with monocytes [4], stimulation of DCs with gamma-irradiated M. tuberculosis H37Rv or bacillus Calmette–Guérin induced significant upregulation of PD-L1 (Figure 1E and 1F). Live Mycobacterium bacilli were more efficient in inducing PD-L1 than killed bacilli, implying that, in addition to cell-wall pathogen-associated molecular patterns, secretory antigens and signals associated with replication of bacteria provide stimuli for the induction of PD-L1. However, we could not detect PD-L2 on DCs (data not shown).

Analysis of polarization of T-cell responses from naive $CD4^+$ T cells revealed that so-called Mycobacterium-educated DCs significantly enhanced the frequency of IFN-γ^+ Th1 cells (Figure 1G and 1H). However, it was not associated with the increased quantities of IFN-γ secretion from these $CD4^+$ T cells (Figure 1I), possibly because of negative signaling by PD-L1 on DCs. Therefore, we attempted to confirm this proposition by blocking PD-L1 on DCs. We confirm that blocking mAbs to PD-L1 were functional, as these antibodies quenched even the basal expression of PD-L1 (Figure 1E). Further, in contrast to the results obtained with monocytes [4], blocking PD-L1 on DCs did not significantly alter the frequency of IFNγ^+CD4^+ T cells (Figure 1G and 1H). However, PD-L1 blockade led to significant increase in the quantity of IFN-γ produced by $CD4^+$ T cells (Figure 1I).

It should be noted that to analyze the expression of surface molecules and intracellular T-cell cytokines, Singh et al stimulated peripheral blood mononuclear cells with M. tuberculosis antigens for 48 hours in the presence of brefeldin A, a Golgi transport blocker [4]. For blocking experiments involving PD ligands or receptors, monocyte–T-cell cultures were treated with brefeldin A for 72 hours [4]. However, because brefeldin A is highly toxic to cells if they are treated for longer periods, short-period treatment (duration, typically 4–6 hours) is recommended. Hence, we suggest that the results reported by Singh et al on Mycobacterium-mediated IFN-γ responses need to be judged with caution because of the possible toxic effects of brefeldin A.

Together, these results provide insight on how PD-L1 on innate cells regulates IFN-γ responses to Mycobacterium. However, the functional repercussion of

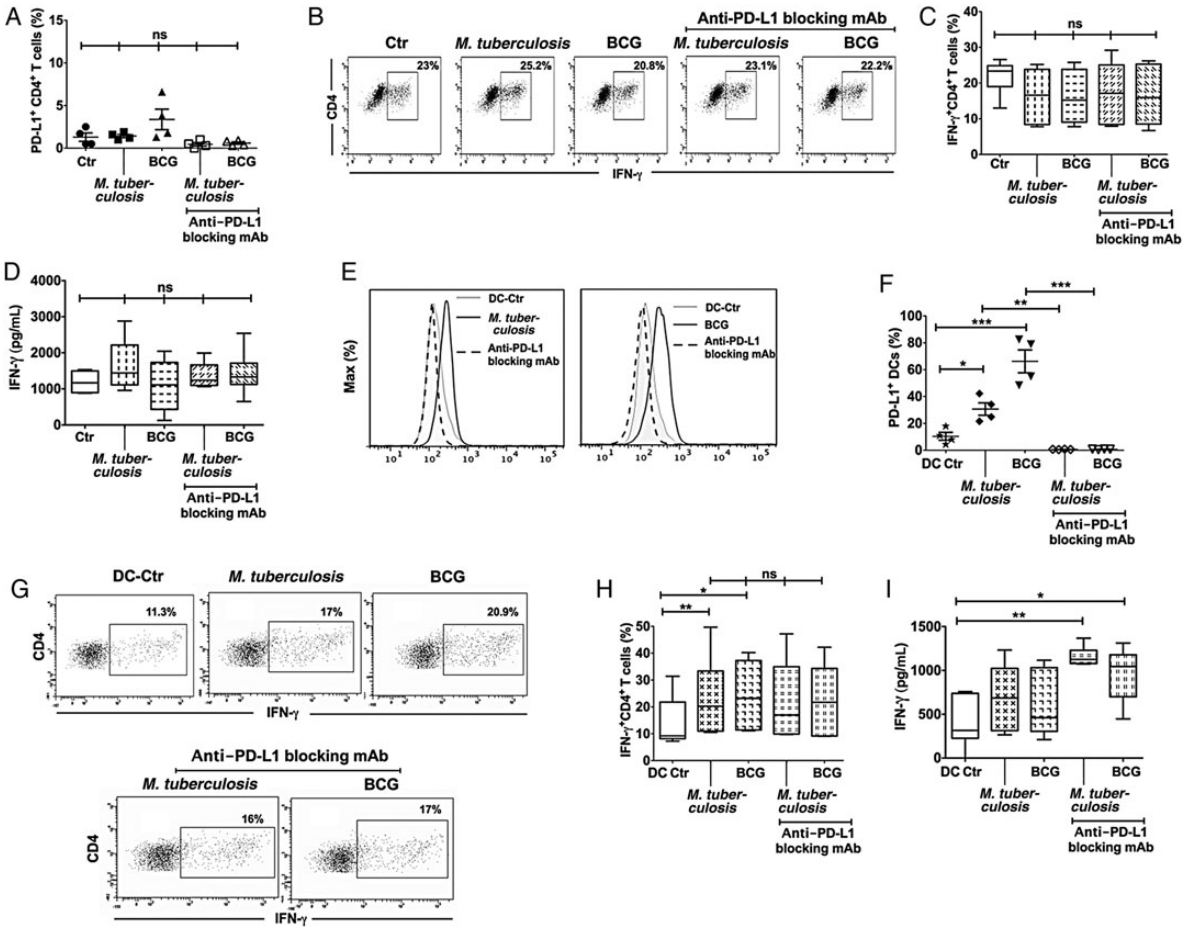

Figure 1. Inhibition of programmed death 1 ligand 1 (PD-L1) on dendritic cells (DCs) enhances Mycobacterium-mediated interferon γ (IFN-γ) production without modulating the frequencies of IFN-γ–producing $CD4^+$ T cells. A, The expression of PD-L1 on activated $CD4^+$ T cells. $CD4^+$ T cells were isolated from buffy coat specimens from healthy donors by using CD4 microbeads (Miltenyi Biotec, France). Permission from the ethics committee was obtained for the use of buffy coats (protocol 12/EFS/079). $CD4^+$ T cells were cultured in 96-well plates at a concentration of 0.1×10^6 cells/well in 200 µL. Cells were stimulated with anti-CD3 and anti-CD28 monoclonal antibodies (mAbs; 1 µg/mL, both from R&D systems, France) alone (Ctr) or with either gamma-irradiated Mycobacterium tuberculosis H37Rv (20 µg/mL) or bacillus Calmette–Guérin (multiplicity of infection, 1:10). The expression of PD-L1 was analyzed by flow cytometry (LSR II, BD Biosciences, France) after 5-day culture by using fluorochrome-conjugated mAbs to PD-L1 (BD Biosciences). To block PD-L1 on $CD4^+$ T cells, blocking mAbs to PD-L1 (10 µg/mL, eBioscience, France) were added 18 hours after Mycobacterium stimulation. The quenching effect of anti–PD-L1 blocking mAbs was analyzed by flow cytometry. Results are mean (± standard error of the mean [SEM]) for 4 independent donors. B–D, The role of PD-L1 on $CD4^+$ T cells in modulating Mycobacterium-mediated IFN-γ responses. The $CD4^+$ T cells were cultured and stimulated as described panel A. After 5 days, cell-free supernatants were collected, and T cells were activated with phorbol myristate acetate (50 ng/mL) and ionomycin (500 ng/mL, Sigma-Aldrich, France), along with GolgiStop (BD Biosciences), for 4 hours. IFN-γ^+CD4^+ T cells were analyzed by flow cytometry. Surface staining was done with fluorochrome-conjugated CD4 mAb (BD Biosciences) and fixable viability dye (eBioscience) to gate and analyze viable $CD4^+$ T cells. Further, cells were fixed, permeabilized (Fix/Perm; eBioscience), and incubated at 4°C with fluorochrome-conjugated mAbs to IFN-γ (BD Biosciences). B, A representative dot plot showing the frequency of $CD4^+$IFN-γ^+ T cells. C, The results from 6 independent donors are expressed using a box and whisker plot, in which boxes represent the interquartile range of data between the 25th and 75th percentiles, whiskers represent the upper and lower limits of the data, and the dividing line in the box represents the median. D, The quantity of IFN-γ (n = 6) in the culture supernatants described above was determined by enzyme-linked immunosorbent assay (ELISA; eBioscience). The results are expressed using a box and whisker plot and the dividing line in the box represents the median. E and F, The expression of PD-L1 on DCs following stimulation with Mycobacterium. Immature DCs (0.5×10^6 cells/mL) derived from peripheral blood monocytes (isolated using CD14 microbeads; Miltenyi Biotec) from healthy donors were cultured in the presence of the cytokines granulocyte-macrophage colony-stimulating factor (1000 IU/10^6 cells) and interleukin 4 (500 IU/10^6 cells; both from Miltenyi Biotec) alone (DC-Ctr) or in the presence of cytokines plus gamma-irradiated M. tuberculosis or bacillus Calmette–Guérin for 48 hours. The expression of PD-L1 was analyzed by flow cytometry. Representative histograms (E) and mean values (± SEM; F) for 4 independent donors are shown. Following Mycobacterium stimulation, DCs were incubated with anti–PD-L1 blocking mAbs for 3 hours, and the quenching effect of blocking mAbs was determined by flow cytometry (E and F). G–I, Inhibition of PD-L1 on DCs enhances Mycobacterium-mediated IFN-γ production without modulating the frequency of IFN-γ–producing $CD4^+$ T cells. DCs were stimulated Mycobacterium and washed extensively. Following incubation with or without anti–PD-L1 mAbs, DCs were cocultured with autologous $CD45RA^+CD25^-$ naive $CD4^+$ T cells (0.1×10^6 cells/well/200 µL) at 1:20 ratios for 5 days. The frequency of $CD4^+$IFN-γ^+ cells was analyzed by flow cytometry. Representative dot blots (G) and pooled data for 6 independent donors are expressed using a box and whisker plot (H). I, The quantity of IFN-γ (n = 6) in the supernatants of DC–$CD4^+$ T-cell cocultures as determined by ELISA. The results are expressed using a box and whisker plot and the dividing line in the box represents the median. *P < .05, **P < .01, and ***P < .001, by 1-way analysis of variance. Abbreviations: BCG, bacillus Calmette–Guérin; ns, not significant.

PD-L1 blockade might depend on the type of innate cells (monocytes vs DCs) and T cells (memory vs naive T-cell polarization). Previous reports have also implicated PD-L1 in the Mycobacterium-mediated expansion of Tregs, the immune suppressor [7, 8]. These data thus provide a rationale for targeting the PD-1–PD-L1 pathway to combat tuberculosis [9, 10].

Notes

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Intravenous immunoglobulin and immune response

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Intravenous immunoglobulin (IVIg) is a pooled preparation of polyspecific, polyreactive immunoglobulin (Ig)G molecules from several thousand healthy donors. The immunoglobulin (Ig) molecule has Fab region, which is involved in antigen-binding and the Fc portion, which is involved in effector function. As IVIg is prepared from multiple donors, it contains numerous antibodies directed against a wide range of antigens; consequently, the variable regions on the Ig Fab fragments in IVIg preparations are diverse. The variable region can bind to non-self-antigens (foreign antigens), self-antigens and anti-idiotypic antibodies. IVIg contains a broad spectrum of antibody specificities against bacterial, viral, parasitic and mycoplasma antigens, that are capable of both opsonization and neutralization of microbes and toxins.

In addition to its initial use as replacement therapy in primary and secondary immunodeficiencies, IVIg is widely indicated in a large spectrum of autoimmune and inflammatory diseases. One of the first proposed mechanisms of action of IVIg was via Fcγ receptor blockade [1]. This study demonstrated that infusion of Fcγ fragments in idiopathic thrombocytopenic purpura/immune thrombocytopenia (ITP) patients increased platelet count, mediated by the blockade of Fcγ receptors [1]. It has since been demonstrated *in vivo* that Fcγ fragments, particularly if sialylated, can exert anti-inflammatory effects [2]. This suggests that the clinical benefits of IVIg may be mediated via an Fc pathway; indeed, to date there have been no studies that demonstrate the clinical benefit of Fab fragments alone.

However, non-Fc mechanisms have been proposed that provide an insight into the possible molecular mechanisms of action of IVIg, although these do not exclude the potential co-operation of Fab and Fc portion of IgG to elicit the effects of IVIg.

One mechanism of action was proposed by Sultan *et al*., who found that anti-idiotypic antibodies in IVIg were effective in the treatment of autoimmune haemophilia [3]. This led to the study and characterization of anti-idiotypic antibodies in IVIg which neutralize pathogenic autoantibodies [4,5]. IVIg was found to contain anti-idiotypes against anti-factor VIII, anti-neutrophil cytoplasmic antibody, anti-DNA, anti-thyroglobulin, anti-acetylcholine receptors and anti-neuroblastoma antigens. Furthermore, anti-idiotypic antibodies have been found to play a role in transplantation due to the anti-human leucocyte antigen antibodies.

In addition to ITP and haemophilia, IVIg has been found to be effective in several inflammatory and autoimmune diseases. Therefore, anti-inflammatory effects of IVIg were studied and were shown to be mediated in part through anti-complement effects. Dermatomyosis is a condition mediated by C5b/C9 membranolytic attack complexes (MACs) in intramuscular capillaries. The formation of MAC occurs when C3 is hydrolyzed into C3b, which leads to the activation of C5b and the formation of MACs. In a study conducted by Basta *et al*. [6], IVIg was found to form complexes with C3, preventing MAC formation and deposition in patients with dermatomyosis. This suggests that the clinical benefit of IVIg can be attributed to complement

scavenging, demonstrating an additional distinct mechanism of action that may be mediated by $F(ab')_2$ and whole IVIg, but not Fcγ fragments alone.

Anaphylatoxins are complement peptides that are produced when the complement system is activated. A study by Basta *et al*. implicates $F(ab')_2$ in the neutralization of anaphylatoxins, such as C3a and C5a [7]. IVIg is able to suppress C3a- and C5a-induced release of thromboxane B2 and histamine, which have proinflammatory properties. Moreover, circulatory collapse caused by C5a was prevented in pigs pretreated with $F(ab')_2$ IVIg. The neutralization of C3a and C5a were observed in cells treated with $F(ab')_2$ IVIg and whole IVIg and not Fcγ IVIg fragments, suggesting that $F(ab')_2$ and not Fcγ are implicated in this process.

IVIg has also been found to be beneficial in a murine model of brain ischaemia and stroke, via a complement scavenging mechanism. Administration of IVIg, either prior to an ischaemic event or during reperfusion, led to a two- to three-fold improvement in functional outcomes in ischaemia and reperfusion. C3 levels were higher in injured compared to non-injured brain regions. Furthermore, compared with wild-type mice, C5-deficient mice were protected from ischaemia and reperfusion. IVIg decreased C3 and caspase 3 activation, suggesting that IVIg inhibits complement-mediated cell damage via scavenging of complement proteins to elicit beneficial effects [8]. In addition to a role in scavenging complement in inflammatory and immune diseases, IVIg has also been shown to alter the cytokine network and mediate the balance between T helper (Th) types. Th cells can be classified into several subsets, such as Th1, Th2, Th17 and regulatory T cells, which produce distinct cytokines. Th1 cells produce cytokines such as interferon (IFN)-γ and tumour necrosis factor (TNF)-α, Th2 cells produce IL-4, IL-5, IL-13 and IL-10, Th17 cells produce IL-17, IL-21 and IL-22, and regulatory T cells which are immunosuppressor cells produce TGF-β and IL-10. In a study conducted by Ruiz de Souza *et al*., peripheral blood monocytes treated with IVIg induced an up-regulation of anti-inflammatory cytokine IL-1 receptor antagonist and down-regulation of several proinflammatory cytokines [9]. By the early 2000s there was an increasing focus on the role of dendritic cells and their effect on T cell polarization. Mature dendritic cells can stimulate naive T helper cells (Th0) and polarize them into distinct subsets. Our study demonstrated that both the $F(ab')_2$ and Fc fragments of IVIg are capable of inhibiting the differentiation and maturation of dendritic cells, suggesting that IVIg is capable of inducing tolerogenic dendritic cell phenotypes [10].

As a consequence of IVIg-induced tolerogenic dendritic cells, regulatory T cells are up-regulated. Using a murine model of autoimmune encephalomyelitis (EAE), prophylactic IVIg was found to increase $CD4^+CD25^+$forkhead box protein 3 ($FoxP3^+$) regulatory T cells [11]. This proliferation of regulatory T cells has also been observed in humans following high-dose IVIg treatment in patients with autoimmune rheumatic disease [12].

We recently reported that, in EAE mice, IVIg inhibits the differentiation of $CD4^+$ T cells to Th1 and Th17 cells [13]. The down-regulation of Th1 and Th17 cells was observed with a concomitant up-regulation of regulatory T cells, demonstrating the reciprocal regulation mechanism of IVIg. Furthermore, the reciprocal regulation was suggested to be $F(ab')_2$-dependent due to the comparable inhibition of Th1 and Th17 cells observed in mice treated with $F(ab')_2$ fragments or IVIg [13].

IVIg-induced expansion of regulatory T cells may be due to several mechanisms. Mazer *et al*. propose that IVIg renders dendritic cells tolerogenic via its interaction with dendritic cell immunoreceptor (DCIR) [14]. This leads to increased levels of $FoxP3^+$ regulatory T cells which can attenuate autoimmune disease severity. Another mechanism of action for regulatory T cell expansion is provided recently by our group. Our report suggests that IVIg-induced expansion of regulatory T cells is due to the induction of cyclo-oxygenase 2-dependent prostaglandin E_2 production in dendritic cells [15]. Inhibition of cyclo-oxygenase 2 enzymatic activity significantly reduced IVIg-mediated regulatory T cell expansion both *in vitro* and *in vivo* in EAE mice. This mechanism was dependent on Fab fragments of IVIg but not Fc.

Immunomodulatory mechanisms of IVIg in autoimmune conditions are not fully understood, although several mutually non-exclusive effects have been proposed. Individually, each of these mechanisms may participate to a certain extent in the overall effect of IVIg. While some of the effects may rely upon the binding of the Fc moiety of IgG to Fcγ receptors on target cells, others may be primarily dependent on the range of variable regions of IgG.

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Disclosure

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LETTERS

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Interferon-a inhibition by intravenous immunoglobulin is independent of modulation of the plasmacytoid dendritic cell population in the circulation: comment on the article by Wiedeman et al

To the Editor:

High-dose intravenous immunoglobulin (IVIG) is used in the therapy of various rheumatic diseases, and the beneficial effects of IVIG in these autoimmune and inflammatory conditions are mediated through several mutually nonexclusive mechanisms (1–3). Recent data reported by Wiedeman et al (4) suggest that one such action of IVIG comprises inhibition of interferon-a (IFNa) production by two distinct mechanisms. The first mechanism described by Wiedeman and colleagues involved Fc-mediated inhibition of immune complex binding to Fcμ receptor IIa on plasmacytoid dendritic cells (PDCs). The second mechanism involved $F(ab')_2$ fragment–dependent inhibition of IFNa production when PDCs were stimulated with Toll-like receptor 7 (TLR-7) and TLR-9 agonists. Those authors also reported that the inhibitory effect of IVIG on IFNa production by TLR-stimulated PDCs required monocyte-derived prostaglandin E_2 (PGE_2) (4). These data, along with findings described in a previous report on the inhibitory effect of IVIG on IFNa-mediated differentiation of monocyte-derived DCs (5), suggest that IVIG affects IFNa-mediated inflammatory pathways. The inhibitory effect of IVIG on IFNa production reported by Wiedeman et al also raises another possibility, that this inhibition might be due to a reduction in the number of PDCs, the principal producers of IFNa.

PDCs and type I IFN are implicated in the pathogenesis of various rheumatic diseases, including systemic lupus erythematosus, myositis, rheumatoid arthritis, and psoriasis (6,7). Aberrant activation of PDCs and their migration to inflamed tissue, and high levels of type I IFN, are hallmarks of these diseases. Ablation of PDCs in vivo was found Heparinized blood samples were obtained from 9 patients with myositis (7 female and 2 male; ages 27–70 years), before and 48–72 hours after initiation of IVIG (1 gm/kg). All patients provided written informed consent for participation in the study, and ethics committee permission was received prior to study initiation. The specific diagnoses of the patients were as follows: polymyositis (n = 3), dermatomyositis (n = 1), anti–signal recognition particle–associated necrotizing myopathy (n = 2), anti–3-hydroxy-3-methylglutaryl-coenzyme A reductase–associated necrotizing myopathy (n = 2), and anti-Mi2–associated unclassified myositis (n = 1). Additional treatments patients were receiving included methotrexate and

to inhibit autoimmunity via expansion of myeloid-derived suppressor cells (8). In addition, antiinflammatory agents, such as cortico- steroids in high doses (1 gm/day), are known to reduce the number of circulating PDCs (9). We therefore investigated whether the inhibitory effects of IVIG on IFNa production reported by Wiedeman et al also implicate modulation of the circulating PDC population in vivo in patients with rheumatic disease.

prednisone. PDCs in whole blood were analyzed by flow cytometry using surface expression of HLA–DR and CD123 (Figure 1A).

Before IVIG therapy, the mean ± SD percentage of circulating PDCs among total blood leukocytes in the myositis patients was 0.104 ± 0.132%. After IVIG therapy, we observed a marginal increase in PDCs in 4 of the patients, probably indicating the inhibitory effects of IVIG on the migration of PDCs toward inflamed tissue. However, overall, IVIG therapy did not lead to significant alterations in circulating PDC

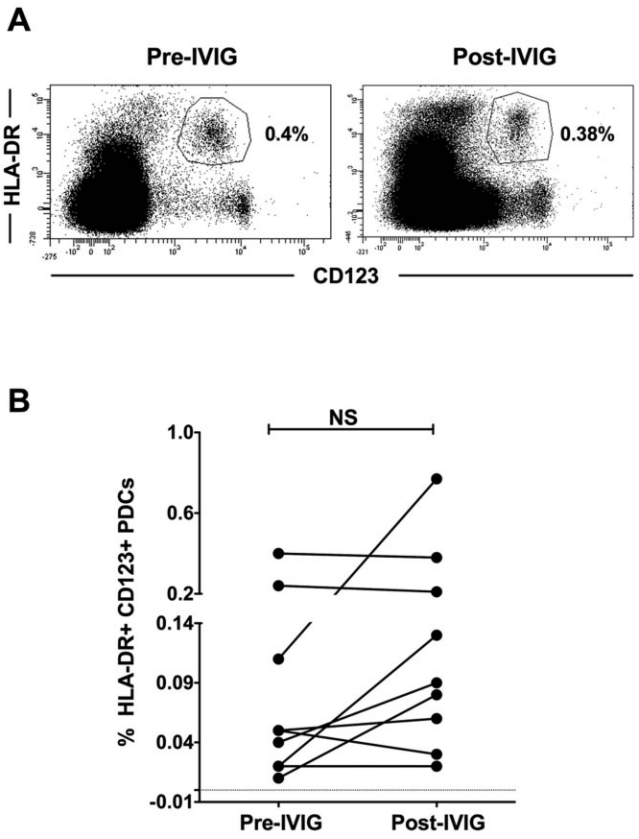

Figure 1. Effect of intravenous immunoglobulin (IVIG) on circulating plasmacytoid dendritic cells (PDCs) from patients with myositis. Heparinized blood samples were obtained 48–72 hours after initiation of high-dose IVIG therapy. Red blood cells were separated from nucleated cells using HetaSep (StemCell Technologies) (1 part HetaSep, 5 parts blood). **A,** Representative dot plots showing the percentage of PDCs with positive gating for HLA–DR and CD123. **B,** Changes in the percentage of HLA–DR+CD123+ PDCs in the circulation of myositis patients (n = 9) following IVIG therapy. Each symbol represents an individual patient. PDCs were analyzed by flow cytometry (LSR II; BD Biosciences) using fluorescence-conjugated monoclonal antibodies to HLA–DR (BD Biosciences) and CD123 (eBioscience). Statistical significance was assessed by Student's paired 2-tailed *t*-test. NS = not significant.

numbers, which were a mean ± SD of 0.197 ± 0.242% of total leukocyte numbers after treatment ($P = 0.249$) (Figure 1B). These data, along with those reported by Wiedeman et al (4), thus suggest that although IVIG inhibits IFNa production from PDCs via monocyte-derived PGE_2, this reduction in IFNa production is not due to an alteration in the number of circulating PDCs in vivo. Importantly, it has been shown that IVIG could also induce cyclooxygenase 2–dependent PGE_2 from human DCs (10), which would lead to an expansion of CD4+CD25+FoxP3+ Treg cells.

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Reply

To the Editor:

Sharma et al present the results of their studies on the effect of IVIG on circulating PDCs in patients with myositis. Based on our reported finding that IVIG inhibits PDC production of IFNa in vitro, they offer two possible hypotheses on how IVIG may affect PDCs in vivo. One possibility is that IVIG treatment would simply reduce PDC numbers. Alternatively, IVIG inhibition of PDCs may reduce their activation and subsequent migration to inflamed tissue, thus resulting in increased numbers of PDCs in the periphery. By comparing the percentage of PDCs in peripheral blood before, and then 2–3 days after, high-dose IVIG therapy, they found that the peripheral PDCs were slightly, but not statistically significantly, increased. These results indicate that IVIG does not induce cell death of PDCs.

We find these results of interest as they demonstrate what we would expect to see in vivo based on our observation that IVIG alters the functional properties of PDCs. We also considered death of PDCs as a potential mechanism by which IVIG could inhibit IFNa production. However, we found that IVIG treatment of lupus immune complex–stimulated PDCs did not increase cell death after 22 hours of culture (Figures 1A and B). As reported in our article, we had shown that in response to TLR ligand stimulation of IFNa, the sialylated subset of IVIG (sialic acid–specific *Sambucus nigra* agglutinin positive) was a more potent inhibitor. Even so, treatment with this IVIG subset did not result in increased PDC death in vitro (Figure 1C). These results are consistent with the maintenance of PDC numbers after IVIG treatment in vivo observed by Sharma et al.

While the number of peripheral PDCs is unaltered with IVIG treatment, it would be of great interest to determine whether high-dose IVIG regulates IFNa production in vivo. Increased serum IFNa has been linked to both myositis and systemic lupus erythematosus, and implicated in their pathogenesis (1,2). It would be relatively straightforward to test

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Intravenous immunoglobulin-induced IL-33 is insufficient to mediate basophil expansion in autoimmune patients

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Intravenous immunoglobulin (IVIg) is used in the therapy of various autoimmune and inflammatory diseases. Recent studies in experimental models propose that anti-inflammatory effects of IVIg are mainly mediated by a2,6-sialylated Fc fragments. These reports further suggest that a2,6-sialylated Fc fragments interact with DC-SIGN[1] cells to release IL-33 that subsequently expands IL-4-producing basophils. However, translational insights on these observations are lacking. Here we show that IVIg therapy in rheumatic patients leads to significant raise in plasma IL-33. However, IL-33 was not contributed by human DC-SIGN[1] dendritic cells and splenocytes. As IL-33 has been shown to expand basophils, we analyzed the proportion of circulating basophils in these patients following IVIg therapy. In contrast to mice data, IVIg therapy led to basophil expansion only in two patients who also showed increased plasma levels of IL-33. Importantly, the fold-changes in IL-33 and basophils were not correlated and we could hardly detect IL-4 in the plasma following IVIg therapy. Thus, our results indicate that IVIg-induced IL-33 is insufficient to mediate basophil expansion in autoimmune patients. Hence, IL-33 and basophil-mediated anti-inflammatory mechanism proposed for IVIg might not be pertinent in humans.

Intravenous immunoglobulin (IVIg) is a therapeutic preparation of normal pooled immunoglobulin G (IgG) obtained from the plasma of several thousand healthy donors. High-dose IVIg (1 – 2 g/kg) is widely used in the treatment of various autoimmune and inflammatory diseases including Kawasaki disease, idiopathic thrombocytopenic purpura, Guillain-Barre´ syndrome, chronic inflammatory demyelinating polyneuropathy, myasthenia gravis, autoimmune blistering diseases, inflammatory myopathies, graft versus host disease and others[1–4]. The cellular and molecular mechanisms of action of IVIg in these diverse diseases remain incompletely understood. However, available evidence both from experimental and clinical studies provide an indicator that IVIg could benefit these diverse diseases via several mutually non-exclusive mechanisms[2,5–10]. These mechanisms include inhibition of activation and functions of innate immune cells such as dendritic cells (DCs), monocytes, macrophages and neutrophils; inhibition of pathogenic effector T cells such as Th1 and Th17 cells; expansion of regulatory T cells (Tregs); modulation of B cell responses; and inhibition of complement pathways. In addition, IVIg has been shown to inhibit inflammatory cytokines and to augment anti-inflammatory molecules such as IL-10 and IL-1 receptor antagonist[11–21].

IgGs are glycoproteins and contain fragment antigen-binding (Fab) regions that recognize antigens, and fragment crystallizable (Fc) regions that exert effector functions upon binding to Fcc receptors. The Fc fragments are glycosylated at Asn297 and recent studies in animal models advocate that anti-inflammatory effects of IVIg

are mediated by a small fraction of antibodies that contain terminal a2,6-sialylated glycans at Asn297. It was proposed that a2,6-sialylated Fc fragments interact with dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin-positive (DC-SIGN[1]) innate cells to release IL-33, which subsequently expands IL-4-producing basophils[22]. However, translational insights on these observations are lacking. Therefore, we investigated whether high-dose IVIg therapy induces IL-33 production in autoimmune patients, which in turn would mediate basophil expansion and IL-4 responses.

Results

IVIg therapy induces IL-33 in autoimmune patients. Previous work on the role of IL-33 in IVIg-mediated anti-inflammatory effects was performed in K/BxN serum-induced murine arthritis model. It should be noted that IVIg is not recommended for rheumatoid arthritis due to its inefficacy to relieve inflammation[4]. Therefore, K/BxN serum-induced murine arthritis model might not provide factual image of the mechanisms of IVIg in autoimmune patients. Earlier studies have indicated that IVIg therapy benefits patients with inflammatory myopathies[1,4]. Therefore, by using heparinized blood samples of these patients (cohort 1 patients), we first investigated the repercussion of IVIg therapy on the induction of IL-33. We found that, out of nine patients, six had minimal level of plasma IL-33 prior to IVIg therapy. The pre-IVIg plasma level of IL-33 was in the range of 150.75 6 79.52 pg/ml (n 5 9) (Fig. 1a). Following IVIg therapy, with an exception of one patient, all remaining patients had significant raise in plasma IL-33 and was in the range of 492.23 6 130.30 pg/ml (n 5 9) (Fig. 1a). However, the increase in IL-33 following IVIg therapy was heterogeneous and was varying from 1.2 to 911-fold.

To confirm these results, we analyzed the plasma samples from another cohort of patients with inflammatory myopathies (n 5 4) or anti-neutrophil cytoplasmic antibody-associated vasculitis (n 5 3) (cohort 2 patients). Importantly, these patients also showed significant increase in plasma IL-33 following IVIg therapy (Fig. 1b) thus confirming the results obtained with cohort 1 patients. The pre-IVIg plasma level of IL-33 was 80.43 6 24.93 pg/ml (n 5 7) that increased to 291.58 6 34.40 pg/ml following IVIg therapy. Together, these results indicate that irrespective of pathologies, IVIg therapy in patients leads to increased plasma level of IL-33.

IVIg-induced IL-33 is not associated with an expansion of basophils. Basophils play a crucial role in the induction of Th2 responses[23,24]. Recent data from K/BxN serum-induced murine arthritis model suggest that IVIg-induced IL-33 promotes basophil expansion[22]. Therefore, we investigated changes in the circulating basophils following IVIg therapy in cohort 1 patients. Basophils were identified based on the expression of FceRI and CD203c (Fig. 2a)[25]. In contrast to the results from murine model, we found that IVIg therapy leads to basophil expansion only in two patients who also showed increased plasma level of IL-33 (Fig. 2b). In other patients, basophils were either declined or unaltered. The changes in the proportion of basophils in the circulation following IVIg therapy were not statistically significant. Importantly, the fold-changes in IL-33 and basophils were not correlated (Fig. 2c). Also contrary to previous report[22], we could hardly detect IL-4 in the plasma of patients following IVIg therapy. Thus, these results demonstrate that IVIg therapy in patients does not lead to an expansion of basophils. Of note, a recent data from murine models of collagen antibody-induced arthritis and K/BxN serum transfer arthritis also reveal that therapeutic effect of IVIg is independent of sialylation and basophils[26].

DC-SIGN-positive human innate cells do not produce IL-33 upon IVIg exposure. DC-SIGN[1] innate cells (or SIGN-R1[1] cells in the murine spleen) were proposed to produce IL-33 upon interaction

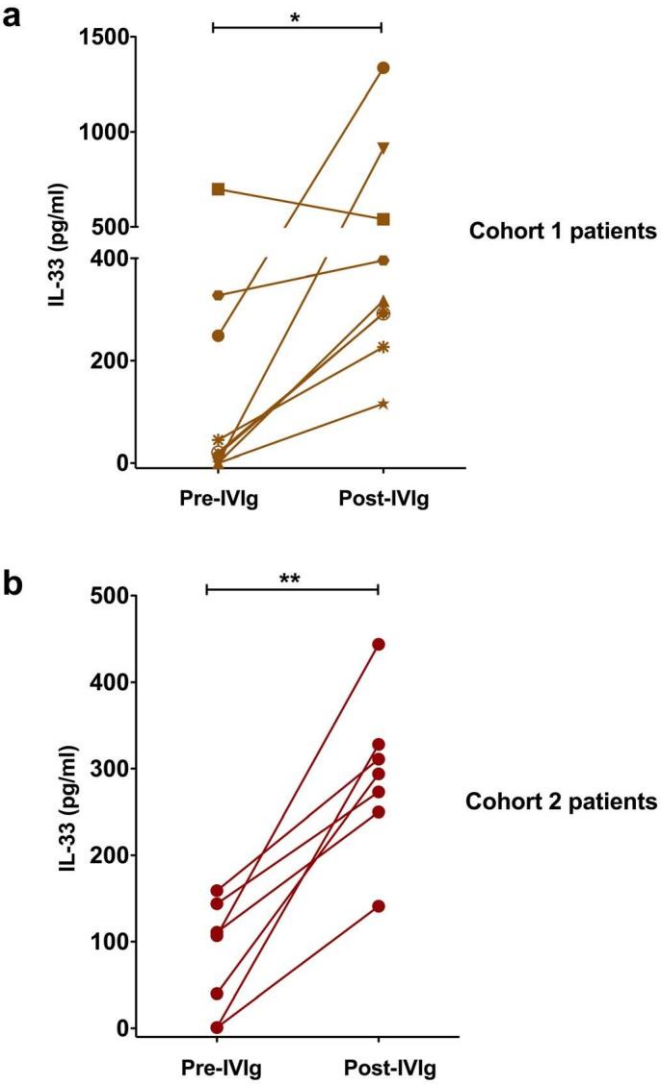

Figure 1 | Consequence of IVIg therapy in autoimmune patients on the plasma level of IL-33. (a) Heparinized blood samples were obtained from nine patients with inflammatory myopathies (Cohort 1 patients) before (Pre-IVIg) and 2-3 days after initiation of IVIg therapy (Post-IVIg). IL-33 (pg/ml) in the plasma was measured by ELISA. Each symbol in the graph represents individual patient. (b) IL-33 in the plasma of four inflammatory myopathies and three anti-neutrophil cytoplasmic antibody-associated vasculitis patients (Cohort 2 patients) before and post-IVIg therapy. The statistical significance as determined by two-tailed Student-t-test is indicated, where *, $P < 0.05$; **, $P < 0.01$.

with a2,6-sialylated Fc fragments of IVIg[22]. By generating humanized DC-SIGN-transgenic mice, the authors found that these transgenic mice express DC-SIGN on DCs, macrophages and monocytes in the blood, bone marrow and spleen. Importantly, higher percentage of monocytes in these transgenic mice expressed DC-SIGN[22].

We analyzed the expression of DC-SIGN in human myeloid cells. Contrary to humanized DC-SIGN-transgenic mice, circulating human monocytes did not express DC-SIGN whereas its expression on macrophages was restricted to M2 type macrophages wherein up to 28% cells were positive for DC-SIGN. We could observe high expression of DC-SIGN (<100%) only in monocyte-derived DCs (Mo-DCs) (Fig. 3a). In the human spleen, up to 24% splenocytes were positive for DC-SIGN (Fig. 3b).

Therefore, we explored if Mo-DCs secrete IL-33 upon IVIg treatment. In contrast to proposition by Ravetch and colleagues, we could

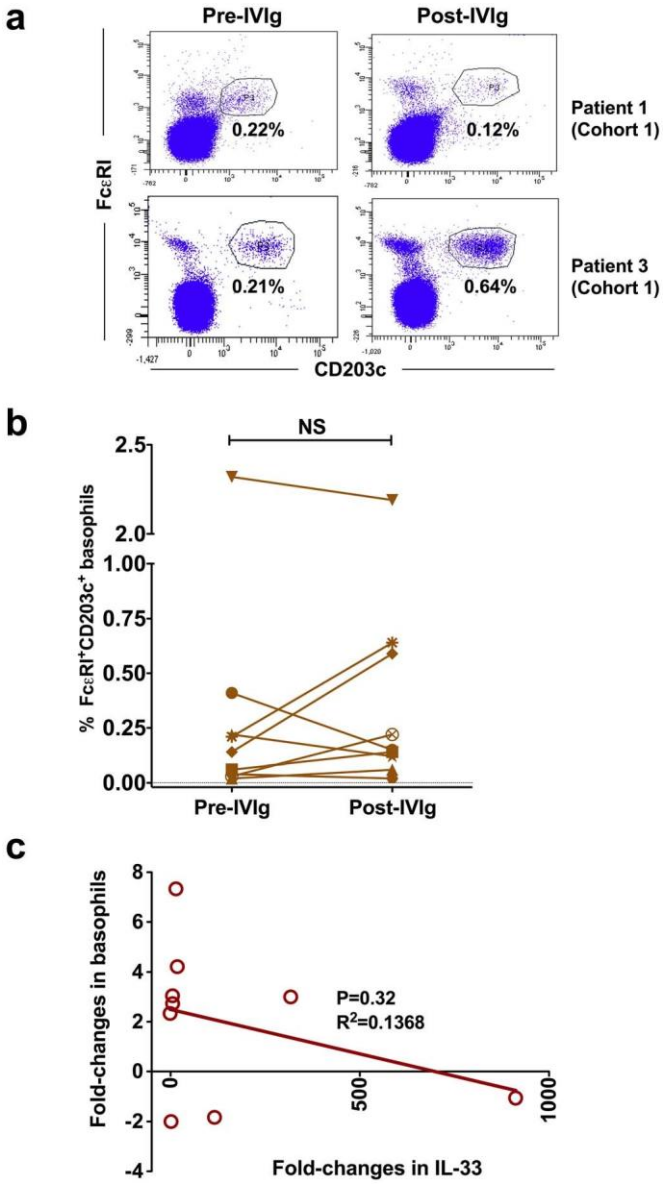

Figure 2 | Changes in the proportion of circulating basophils of autoimmune patients following IVIg therapy. Heparinized blood samples were obtained from cohort 1 patients with inflammatory myopathies before (Pre-IVIg) and 2 – 3 days after initiation of IVIg therapy (Post-IVIg). (a) Representative dot-plots showing basophils from cohort 1 patients gated positive for FceRI and CD203c (b) Modulation of circulating basophils following IVIg therapy (n 5 9). Basophils were analyzed in the whole blood by flow cytometry. The statistical significance as determined by two-tailed Student-t-test is indicated, where NS, non-significant. (c) The correlation between fold-changes in IL-33 and basophils following IVIg therapy.

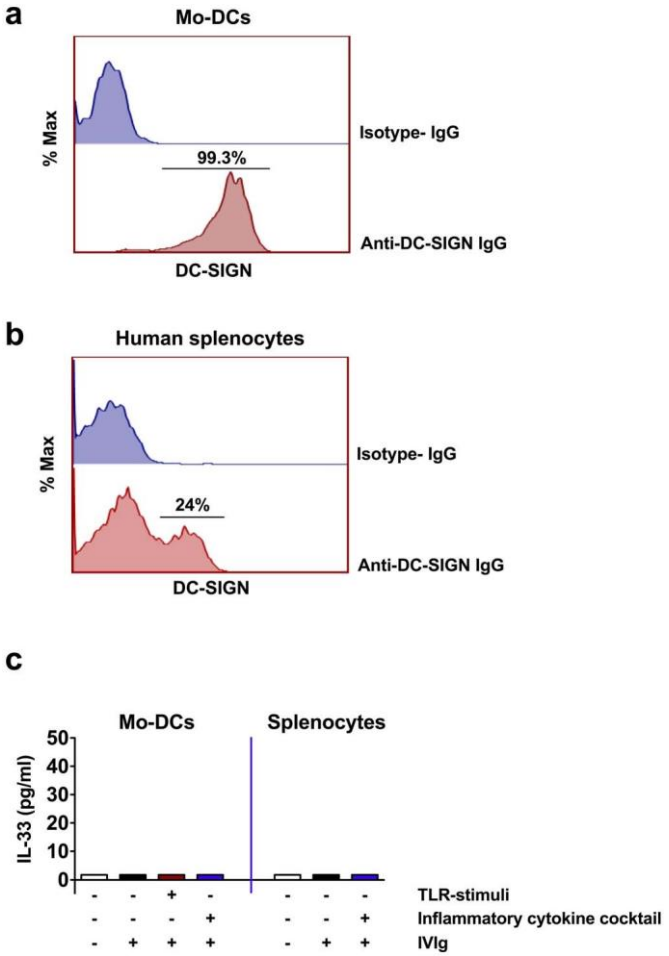

Figure 3 | Effect of IVIg on the IL-33 production from DC-SIGN^1 human innate cells. (a and b) Histograms showing the expression of DC-SIGN by healthy donor's monocyte-derived human dendritic cells (Mo-DCs) and splenocytes. (c) IVIg does not induce IL-33 from DC-SIGN^1 human innate cells. Mo-DCs or human splenocytes (n 5 5 donors) were exposed to IVIg either under non-inflammatory conditions or under inflammatory conditions (TLR-stimuli or inflammatory cytokine cocktail) for 48 hours. IL-33 in the culture supernatants was analyzed by ELISA.

detect secreted IL-33 from IVIg-exposed DC-SIGN^1 Mo-DCs neither under non-inflammatory nor under inflammatory conditions (Fig. 3c). Similarly, despite the presence of DC-SIGN^1 cells in the spleen, human splenocytes did not produce detectable levels of IL-33 upon IVIg exposure both under inflammatory and non-inflammatory conditions (Fig. 3c).

Discussion

Our results demonstrate that IVIg therapy induces IL-33 in autoimmune patients thus confirming the previous observation made in mice. However, IL-33 was not contributed either by splenic DC-SIGN^1 cells or myeloid DCs. Also, the amount of IL-33 induced in the patients was not sufficient to expand basophils. It should be noted that the quantity of IL-33 protein induced in the mice following IVIg treatment was not presented in the previous report. In addition, significant amount of data on IVIg was indirect rather than direct demonstration of IVIg-mediated regulation of cytokine network[22]. Authors showed that IVIg induces about 12-fold increase in IL-33 mRNA level. However, the contribution of this increased IL-33 mRNA towards IL-33 protein is not clear. Considering five liters as total blood volume in adults, our results show that IVIg induces $<2460 \pm 650$ ng of IL-33 (based on the data from cohort 1 patients). However, to demonstrate the role of IL-33 in IVIg-mediated anti-inflammatory effects, Anthony *et al.*, injected 400 ng of IL-33 for four consecutive days[22]. As mouse weighing 25 g would have <1.5 ml of blood, based on the IL-33 data from patients, we could infer that the amount of exogenous IL-33 injected into the mice represents at least 540-times excess of IL-33 that otherwise induced by IVIg. This might explain why IVIg failed to induce expansion of basophils in the patients. Although in our study, patients' sample size was small, we included diseases such as inflammatory myopathies and vasculitis that were shown to benefit from IVIg therapy. Further investigations in a larger number of patients should confirm these observations.

Table 1 | Summary of data for autoimmune rheumatic patients

Cohort 1 patients

Number	Disease	Age (years)	Sex	IVIg	Additional treatments
1	Polymyositis	59	F	CLAIRYGH 1 g/kg	Methylprednisolone
2	Anti-SRP associated necrotizing myopathy	27	F	CLAIRYGH 1 g/kg	Prednisone, Methotrexate
3	Anti-HMGCR associated necrotizing myopathy	62	F	CLAIRYGH 0.5 g/kg	Prednisone, Methotrexate
4	Anti-HMGCR associated necrotizing myopathy	61	F	CLAIRYGH 1 g/kg	Prednisone, Methotrexate
5	Dermatomyositis	52	F	CLAIRYGH 1 g/kg	Prednisone, Methotrexate
6	Polymyositis associated with mixed connective tissue disease and Sjögren's syndrome	41	F	CLAIRYGH 1 g/kg	Prednisone, Methotrexate
7	Anti-SRP associated necrotizing myopathy	40	M	CLAIRYGH 1 g/kg	Prednisone, Methotrexate
8	Anti-Mi2 associated unclassified myositis	30	M	CLAIRYGH 1 g/kg	Prednisone, Methotrexate
9	Polymyositis and probable associated Sjögren's syndrome	70	F	CLAIRYGH 1 g/kg	Prednisone, Methotrexate

Cohort 2 patients

Number	Disease	Age (years)	Sex	IVIg	Additional treatments
1	Dermatomyositis	22	F	TEGELINEH 1g/kg	Prednisone, Mycophenolate mofetil
2	Polymyositis	42	M	TEGELINEH 1g/kg	Prednisone, Methotrexate
3	Dermatomyositis	35	M	TEGELINEH 1g/kg	Prednisone
4	Polymyositis	46	F	TEGELINEH 1g/kg	Prednisone, ciclosporin
5	Microscopic polyangiitis	61	F	TEGELINEH 1g/kg	Prednisone
6	Wegener's granulomatosis	62	M	TEGELINEH 1g/kg	None
7	Microscopic polyangiitis	61	M	TEGELINEH 1g/kg	Prednisone, Mycophenolate mofetil

SRP, Signal Recognition Particle; HMGCR, 3-hydroxy-3-methylglutaryl-CoA reductase.

The role of Fc-sialylation, DC-SIGN and Fcc receptor IIB (FccRIIB) in the anti-inflammatory effects of IVIg has been debated recently by several groups[27]. Mice and humans show wide variations in the expression pattern of FccRs, and the phenotype and anatomical distribution of innate cells. Unlike mice, human innate cells express both activating FccRIIA and inhibitory FccRIIB. Therefore, the proposition that IVIg enhances FccRIIB on effector macrophages of mice without having corresponding data on FccRIIA might provide a biased picture on the mechanisms of IVIg. Gene array analysis could not confirm IVIg-mediated up-regulation of FccRIIB in the patients with Kawasaki disease[28]. In line with this report, another recent study failed to demonstrate enhanced expression of FccRIIB on monocytes following IVIg therapy in children with immune thrombocytopenia[29]. Also, FccR polymorphisms did not predict response to IVIg in myasthenia gravis[30]. Although DC-SIGN promoter 2336 A/G (rs4804803) polymorphism was associated with susceptibility of Kawasaki disease, this variant was found to be not associated with the occurrence of IVIg resistance[31]. Of note, treatment response in Kawasaki disease is apparently associated with sialylation levels of endogenous IgG but not therapeutic IVIg[32]. All these data thus questions the relevance of DC-SIGN-FccRIIB pathway of anti-inflammatory mechanisms of IVIg in humans.

Several recent studies have challenged the concept of a2,6-sialylated Fc fragments-mediated anti-inflammatory mechanism of IVIg both in experimental models and in humans. IVIg could inhibit human Th17 cell differentiation and expansion independent of antigen presenting cells and hence independent of interaction of DC-SIGN and a2,6-sialylated Fc fragments[13–15]. Also, F(ab')$_2$ fragments of IVIg exerted similar effects thus pointing towards dispensability of a2,6-sialylated Fc fragments in mediating the suppression of Th17 cells. We have demonstrated that DC-SIGN and a2,6-sialylated Fc fragment interaction is dispensable for the anti-inflammatory activity of IVIg on human DCs[33]. F(ab')$_2$ fragments but not Fc fragments of IVIg were shown to mediate Treg expansion by inducing cyclooxygenase-2-mediated prostaglandin E2 secretion in human myeloid DCs and was dependent in part on DC-SIGN[19]. Similarly, sialylation-enriched F(ab')$_2$ fragments could inhibit interferon-a production from toll-like receptor (TLR)7 and TLR9 stimulated human plasmacytoid DCs, although sialic acid itself was not required[34].

In the previous reports, Ravetch and colleagues enriched sialic acid-containing IgG-Fc by using sialic acid-specific lectin ***Sambucus nigra*** agglutinin-based affinity fractionation[22,35–37]. However, by using same fractionation method, Guhr ***et al.***, showed that IVIg fractions depleted for the sialylated antibody fraction exert benefits in a murine model of passive-immune thrombocytopenia similar to that of intact IVIg. However, sialic acid-enriched IVIg fraction failed to enhance platelets count in this model[38]. Similar sialic-acid independent anti-inflammatory mechanisms were also reported in murine herpes simplex virus encephalitis model[39]. Further, Kaßermann and colleagues showed that lectin fractionation of IVIg results in increased sialylation of Fab fragments but not Fc fragments. By using human whole blood stimulation assay either with lipopolysaccharide or phytohaemagglutinin, they further showed that anti-inflammatory effects of IVIg is associated with F(ab')$_2$ fraction of IVIg[40]. In animal models of immune thrombocytopenia and multiple sclerosis, the beneficial effects of IVIg were independent of Fc or F(ab')$_2$-sialylation and FccRIIB[41–44]. Based on these results, it was suggested that genetic background of the mice and dose of IVIg are the critical factors that determine the role of FccRIIB in IVIg-mediated beneficial effects. In line with these observations, two studies have failed to demonstrate the direct interaction between sialylated IgG Fc fragment and DC-SIGN[45,46]. These data thus point out that a2,6-sialylated Fc fragment-DC-SIGN-FccRIIB mechanism merely represents one of the several anti-inflammatory mechanisms of IVIg that were reported. Therefore, this anti-inflammatory pathway of IVIg might be operational in certain pathologies and experimental models and might not be considered as a universal mechanism.

It was proposed that in humanized DC-SIGN-transgenic mice, DC-SIGN[1] innate cells such as monocytes, macrophages and DCs produce IL-33 upon interaction with a2,6-sialylated Fc fragments of IVIg[22]. Recent reports show that IL-33 is an important player for the promotion of Th2 responses and activated DCs are one of the sources of this cytokine[47,48]. We found that unlike monocytes from huma-

nized DC-SIGN-transgenic mice that were highly positive for DC-SIGN, human monocytes hardly express DC-SIGN. Further, human Mo-DCs despite expressing DC-SIGN, failed to produce IL-33 when exposed to IVIg either under non-inflammatory or inflammatory conditions. In wild type mice, it was suggested that a2,6-sialylated Fc fragments bind to SIGN-R1 expressed on splenic marginal zone macrophages[35]. Marginal zone macrophages are absent in human spleen and data from humans show that spleen is dispensable for the anti-inflammatory effects of IVIg. In line with this concept, by using a passive model of induced immune thrombocytopenia, it was shown that IVIg is fully functional in splenectomized mice although this report supported the sialic acid and SIGN-R1-dependent mechanisms of IVIg[49]. We found that despite the presence of DC-SIGN^{1} innate cells in the human spleen, IVIg could not induce IL-33 from the splenocytes. All these data indicate that spleen and DC-SIGN^{1} cells are dispensable for IVIg-mediated IL-33 induction in humans. Thus, the source of IL-33 in humans following IVIg therapy remains elusive. As IVIg is known to cause apoptosis of cells, we suggest that secondary necrosis of late stage apoptotic cells could release IL-33[50–52]. This process might depend on the signals provided by anti-Fas IgG or anti-Siglec IgG in the IVIg preparations rather than the repercussion of interaction of a2,6-sialylated Fc fragments with DC-SIGN[53,54]. In addition, IL-33 is also constitutively expressed in the nucleus of endothelial cells and epithelial cells *in vivo*[55].

Methods

Patients. All experiments were performed in accordance with relevant guidelines and regulations. We obtained heparinized blood samples of nine patients (cohort 1 patients) with inflammatory myopathies (Table 1). Patients were aged 49.1 ± 15.2 years and include two men. Blood samples were obtained before and 2 – 3 days following initiation of IVIg therapy (CLAIRYG®, Laboratoire Français du Fractionnement et des Biotechnologies, France). Informed consent was obtained from all the patients. The study was approved by CPP-Ile-de-France VI, Groupe Hospitalier Pitié-Salpêtrière, Paris. In addition, we also analyzed plasma samples of seven rheumatic patients (cohort 2 patients) before and 2 – 3 days post-IVIg therapy (TEGELINE®, Laboratoire Français du Fractionnement et des Biotechnologies). The patients were aged 47 ± 5.8 years (four men) and include inflammatory myopathies and anti-neutrophil cytoplasmic antibody-associated vasculitis (Table 1).

Analysis of basophils. Red blood cells (RBCs) from heparinized blood samples of cohort 1 patients were depleted by using HetaSepTM (Stemcell Technologies Sarl, France) and nucleated cell suspension was obtained. Basophils were analyzed in RBC-depleted cell suspension by flow cytometry (LSR II, BD Biosciences, France) using fluorochrome-conjugated monoclonal antibodies to FceRI (Miltenyi Biotec, France) and CD203c (eBioscience, France). Data were analyzed by FACSDiva™ software (BD Biosciences).

Generation of monocyte-derived DCs. Buffy coats from the healthy donors were purchased from Centre Necker-Cabanel, Etablissement Français du Sang (EFS), Paris, France. Institut National de la Santé et de la Recherche Médicale-EFS ethical committee permission (Nu12/EFS/079) was obtained for the use of buffy coats of healthy donors. Peripheral blood mononuclear cells (PBMCs) were purified from the buffy coats by density gradient centrifugation using Ficoll-paque PREMIUM (GE healthcare, France). CD14^{+} monocytes were isolated from PBMCs by using CD14 microbeads (Miltenyi Biotec). Purified monocytes were then cultured for 6 days in RPMI-1640 medium plus 10% fetal calf serum (FCS) containing cytokines GM-CSF (1000 IU/10^{6} cells) and IL-4 (500 IU/10^{6} cells) (both from Miltenyi Biotec) to obtain DCs[56]. The purity of DCs was >98%. DC-SIGN expression on Mo-DCs was examined by flow cytometry using fluorochrome-conjugated monoclonal antibodies (BD Biosciences) and data were analyzed by FACSDiva™ and FlowJo softwares (Tree Star, USA).

Isolation of human splenocytes. The remnant human spleen sections from individuals submitted for pathological diagnosis were obtained from service d'anatomie pathologique, Hôpital Européen Georges Pompidou, Paris, France. Only healthy spleen tissues were used for the research purpose. Since the study did not require additional sampling, an approval from an ethics committee was not required under French law according to the article L.1121-1 of the public health code. The article states that: The research organized and performed on human beings in the development of biological knowledge and medical research are permitted under the conditions laid down in this book and are hereinafter referred to by the term "biomedical research". The article further states that it does not imply under conditions: For research in which all actions are performed and products used in the usual way, without any additional or unusual diagnostic procedure or surveillance.

The spleen sections were collected in RPMI 1640 medium supplemented with 100 IU/ml penicillin, 100 mg/ml streptomycin, and 10% FCS. Single-cell suspension of splenocytes was obtained by mechanical disaggregation of spleen tissue pieces by using gentleMACS dissociator (Miltenyi Biotec) followed by filtration through 70-mm nylon membrane filter (BD Biosciences). Splenocytes were then subjected to Ficoll-Paque PREMIUM density gradient centrifugation to obtain mononuclear cells. DC-SIGN expression on the splenocytes was investigated by flow cytometry using fluorochrome-conjugated monoclonal antibodies and data were analyzed by FACSDiva™ and FlowJo softwares.

Stimulation of cells. Mo-DCs (0.5 × 10^{6}/ml) were cultured in RPMI 1640-10% FCS containing GM-CSF and IL-4 in a 12-well plate. The cells were then exposed to IVIg (25 mg/ml) for 48 hours to analyze the effect of IVIg on IL-33 production under non-inflammatory conditions. In parallel, Mo-DCs were stimulated with either TLR4 ligand lipopolysaccharide (100 ng/ml/0.5 × 10^{6} cells) (Sigma-Aldrich, France) or inflammatory cytokine cocktail (10 ng/ml each of IL-1b, IL-6 and TNF-a, all from ImmunoTools, Germany)[57]. After four hours, IVIg was added and cultures were maintained for 48 hours to analyze the effect of IVIg on IL-33 production under inflammatory conditions.

Similarly, splenocytes (0.5 × 10^{6}/ml) were cultured in RPMI 1640-10% FCS for 48 hours either alone or with IVIg. In addition, splenocytes were also stimulated with inflammatory cytokine cocktail and IVIg was added to the cultures after four hours. The cultures were maintained for 48 hours.

Quantification of cytokines. IL-33 in the plasma samples of the patients and in cell-free culture supernatants was quantified by ELISA (R&D systems, France). IL-4 in the plasma was also measured by ELISA (R&D systems).

Statistical analysis. Data was analyzed using Prism 5 software (GraphPad software). Two-tailed Student's t-test was used to determine the statistical significance of the data. Values of $P \geq 0.05$ were considered as statistically significant.

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Author contributions

J.B. designed the research, M.S., C.S., P.H., M.S.M., E.S-V., L.G. & M.L. performed the experiments, M.S., P.H., M.S.M., S.V.K. & J.B. analyzed the data, Y.S., L.M. & O.B. provided blood samples of the patients, P.B. provided the spleen tissues, J.B. wrote the paper and all authors reviewed and approved the manuscript.

Additional information

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SHORT COMMUNICATION

Sialylation may be dispensable for reciprocal modulation of helper T cells by intravenous immunoglobulin

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Several mechanisms account for the beneficial effect of intravenous immunoglobulin (IVIg) in autoimmune and inflammatory diseases. These mechanisms include effects on the cellular compartment and on the humoral compartment. Thus, IVIg impacts on dendritic cells, macrophages, neutrophils, basophils, NK cells, and B and T lymphocytes. Several studies have emphasized that the antiinflammatory effect of IVIg is dependent on α2,6-sialylation of the N-linked glycan on asparagine-297 of the Fc portion of IgG. However, recent reports have questioned the necessity of sialylated Fc and the role of FcγRIIB in IVIg-mediated antiinflammatory effects. In view of the critical role played by Th17 cells in several autoimmune pathologies and the increasing use of IVIg in several of these conditions, by using neuraminidase-treated, desialylated IVIg, we addressed whether the α2,6-sialylation of IgG is essential for the beneficial effect of IVIg in experimental autoimmune encephalomyelitis (EAE), a Th17-driven condition, and for the reciprocal modulation of helper T-cell subsets. We observed no difference in the ability of IVIg to ameliorate EAE irrespective of its sialylation. Our findings thus show that sialylation of IVIg is not necessary for IVIg-mediated amelioration of EAE or for downregulation of Th17 cells and upregulation of regulatory T cells.

Keywords: Experimental autoimmune encephalomyelitis · Intravenous immunoglobulin · Regulatory T cells · Sialylation · Th1 · Th17

Additional supporting information may be found in the online version of this article at the publisher's web-site

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Introduction

Intravenous immunoglobulin (IVIg) is a therapeutic preparation of polyspecific human gammaglobulin (IgG) derived from the pooled plasma of thousands of healthy donors [1–3]. IVIg is primarily used as an IgG replacement therapy in immune-deficient patients. Paradoxically, high doses of IVIg are also used to treat a number of autoimmune and inflammatory pathologies including immune thrombocytopenia, Guillain-Barr´e syndrome, Kawasaki disease, and chronic inflammatory demyelinating polyneuropathy [1, 3]. The beneficial effect of IVIg in two opposing clinical scenarios (immunodeficiency and autoimmune pathology) is intriguing; notably the role of IVIg therapy in the latter condition is under intense investigation. IVIg is known to function by several mutually nonexclusive mechanisms modulating both molecular and cellular networks of the immune system [4]. Molecular pathways modulated by IVIg include cytokines, autoantibodies, complement proteins and Fc receptors; cellular targets include antigen-presenting cells (DCs), macrophages, neutrophils, basophils, natural killer cells, B and T lymphocytes [5–8].

Several previous reports have uncovered a surprising role of IgG glycosylation that accounts for the therapeutic efficacy of IVIg [9–12]; it has been demonstrated that the antiinflammatory activity of IVIg is dependent exclusively on α2,6-sialylation of N-linked glycan on asparagine-297 in the Fc portion of IgG [9]. Using a K/BxN model of arthritis, it was shown that the sialylated Fc fraction of IVIg upregulates FcγRIIB (an inhibitory Fc receptor) on the effector macrophages, thus increasing their activation threshold and circumventing the joint damage [9]. However, recent studies have questioned the requirement of sialylated Fc and the role of FcγRIIB in models of immune thrombocytopenia and in IVIg-mediated inhibition of innate immune cell functions [13–19].

Interleukin-17 secreting helper T (Th17) cells have emerged as key pathogenic players in rheumatoid arthritis, antineutrophil cytoplasmic antibody-associated vasculitis, asthma, allergic contact dermatitis, systemic lupus erythematosus, chronic inflammatory bowel disease, and MS [20]. In these conditions, Th17 cells coordinate with IFN-γ-secreting Th1 cells and attract other effector cells to the sites of inflammation. Interestingly, regulatory T (Treg) cells expressing the transcription factor Foxp3 are implicated in the suppression of autoreactive T cells, including Th17 cells and preventing Th17-dependent autoimmune conditions [20].

In view of the importance of the Th17 cells in autoimmune and inflammatory conditions and the proposed role of sialylation of IVIg, in this study, we examined the role of sialylation of IVIg in a Th17 cell mediated autoimmune model, the experimental autoimmune encephalomyelitis (EAE), a classical murine model of MS proven to be mediated by Th17 cells [21]. Our results indicate that the reciprocal regulation of $CD4^+$ T cells by IVIg in EAE appears to be independent of sialylation.

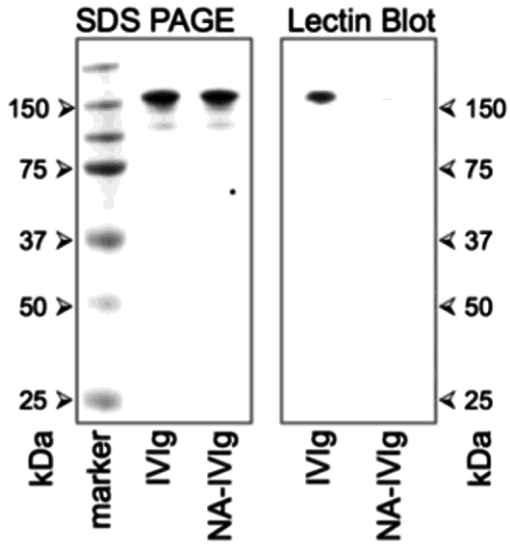

Figure 1. SDS-PAGE and lectin blot profiles of native and desialylated IVIg (NA-IVIg). One microgram of IgG was loaded and run on NuPage 10% BisTris gels under nonreducing conditions. The gels were stained with colloidal Coomassie (left) or blotted onto nitrocellulose, probed with biotin-SNA and AP-streptavidin, and visualized with chromogenic AP conjugate substrate (right). The molecular weight markers are also indicated. Data shown are representative of more than five independent desialylation and purification processes.

Results and discussion

Considering the crucial role played by Th17 cells in many autoimmune pathologies and increasing use of IVIg in several of these conditions, it is important to understand the role of sialylation of IVIg in a Th17 cell mediated autoimmune model. EAE is classical murine model of MS proven to be mediated by Th17 cells [21]. Using myelin oligodendrocyte glycoprotein ($MOG_{35–55}$)-induced EAE, we have recently demonstrated that IVIg delays the onset and decreases severity of disease by inhibiting Th17 and Th1 cells, and concomitantly expanding $Foxp3^+$ Treg cells [22]. To our surprise, this reciprocal regulation was independent of inhibitory FcγRIIB. Here, we addressed whether the α2,6-sialylation on IgG is required for the beneficial effect of IVIg in EAE and for the reciprocal modulation of helper T-cell subsets by using neuraminidase-treated, desialylated IVIg.

Desialylation and integrity of the neuraminidase-treated IVIg (NA-IVIg) was confirmed by SDS-PAGE, and lectin blottings (Fig. 1). Reverse phase high performance liquid chromatography (RP-HPLC) revealed that NA-IVIg contained less than 0.1 mg of sialic acid per gram of IgG as compared native, untreated IVIg (0.67 mg/g of IgG) (data not shown) [14].

EAE was induced in 10-week-old female C57BL/6J mice. NA-IVIg delayed the onset and decreased severity of the disease similar to that of IVIg ($p < 0.001$ for both IVIg and NA-IVIg) (Fig. 2). We observed no difference in the ability to ameliorate EAE between IVIg and desialylated IVIg (Mean maximal score (MMS) ± SD for control = 3.28 ± 0.58; IVIg = 2.04 ± 0.98;

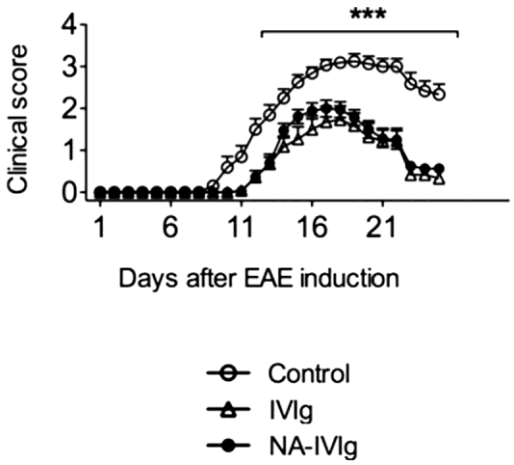

Figure 2. Neuraminidase-treated desialylated IVIg (NA-IVIg) delays the onset of EAE similar to native IVIg. EAE was induced in 10-week-old female C57BL/6J mice. Native IVIg (open triangles) or NA-IVIg (filled circles) was given at 0.8 g/kg from day 0 to 18. Control mice received an equal volume of PBS (open circles). Daily clinical scores of control, IVIg and NA-IVIg groups are shown as mean + SEM of n = 11–18 mice pooled from two independent experiments. *** $p < 0.001$, using two-way ANOVA with Bonferroni's post t test.

NA-IVIg = 2.30 ± 0.60, $p = 0.7124$ between IVIg and NA-IVIg group). Hence, sialylation of IgG is not required for beneficial effect of IVIg in EAE.

Mechanistically, IVIg delays the onset of EAE by inhibiting differentiation of naïve $CD4^+$ T cells into encephalitogenic Th17 and Th1 cells, and expanding $Foxp3^+$ Treg cells. We explored whether desialylated IVIg also exerted similar mechanisms. Consistent with our published results on IVIg [22], NA-IVIg also decreased Th17 cells (fivefold change, $p = 0.015$) and Th1 cells (sevenfold change, $p = 0.015$) in draining lymph nodes (Fig. 3A and C). Accordingly, this inhibition was associated with an increase in the number of $Foxp3^+$ Treg cells in the spleen (1.5 fold change, $p = 0.015$; Fig. 1B and D). Therefore, reciprocal regulation of $CD4^+$ T cells by IVIg in EAE appears to be independent of sialylation. Recent studies from our laboratory showed that $F(ab')_2$ fragments of IVIg could inhibit expansion and activation of human Th17 cells [23] and could induce Treg-cell expansion by inducing COX-2-dependent prostaglandin E2 in DCs [18]. Further, in vitro studies showed that the observed antiinflammatory effects of IVIG are Fab-mediated and not sialic acid-dependent. It was proposed that the skewed antibody repertoire in sialylated IVIg prepared by lectin chromatography is responsible for this effect [19]. Together, these data raise intriguing questions on the role of sialylation of Fc-fragment of IgG for the beneficial effect of IVIg and the reciprocal modulation of helper T-cell subsets by IVIg in murine model of MS.

Concluding remarks

The mechanisms underlying the potent antiinflammatory effect of IVIg in a number of autoimmune and inflammatory diseases have not been completely elucidated. It has been proposed that antiinflammatory activity of IVIg is dependent exclusively on sialylation of the Fc portion of IgG. In the present study, we demonstrate that the protection against EAE by IVIg and reciprocal regulation of proinflammatory encephalitogenic Th17 and Th1 cells and $Foxp3^+$ Treg cells are not dependent on sialylation of IgG. In conclusion, these data along with those reported in murine immune thrombocytopenia further question the role of Fc sialylation in the mechanism of IVIg effect and highlight the importance of multipronged antiinflammatory action of IVIg.

Materials and methods

Desialylation of IVIg with neuraminidase and confirmation by lectin blotting and HPLC

IVIg (Hizentra®) was desialylated by enzymatic digestion as reported previously [14]. Briefly, seven units of recombinant neuraminidase (New England BioLabs, USA) were incubated with each mg IVIg for 48 h at 37°C. Following this, concentration and buffer exchange to PBS was performed by tangential flow filtration using a 100 kD millipore filter unit (minimate TFF system, Pall). To confirm desialylation by lectin blotting, one microgram IgG was loaded and ran on a NuPage 10% Bis-Tris gels under non-reducing conditions (Invitrogen, USA). The gels were stained with colloidal Coomassie (Gelcode, Thermo Scientific, USA) or blotted onto nitrocellulose. The blots were blocked with Carbo-Free blocking solution (Vector Laboratories, USA), probed with biotin-SNA (2 g/L, Vector) and AP-streptavidin (1.5 g/L, Invitrogen) and visualized with chromogenic AP conjugate substrate (BioRad, Switzerland). Alternatively for HPLC, sialic acid was released by acidic hydrolysis of neuraminic acid in 0.25 M $NaHSO_4$ followed by derivatization of the glycan with the fluorophore1,2-diamino-4,5-methylenedioxybenzene dihydrochloride (DMB). Quantification of the derivatized sialic acid was performed by RP-HPLC using *N*-acetyl neuraminic acid (Neu5Ac; Fluka, Switzerland) as a standard and expressed as Neu5Ac per IgG (weight/weight) [14].

Induction EAE and treatment with IVIg or NA-IVIg

Animal experiments were performed as previously described [18, 22] according to the Charles Darwin ethical committee guidance (UPMC Paris). A 10-week-old C57BL/6J mice (Janvier Laboratories, France) were immunized subcutaneously with 200 μL of emulsion (50 μL per site) containing 200 μg of MOG_{35-55} peptide (MEVGWYRSPFSRVVHLYRNGK, PolyPeptide laboratory Strasbourg, France) emulsified in complete Freund's adjuvant (Sigma–Aldrich) containing 880 μg of nonviable *Mycobacterium tuberculosis* antigen H37RA (Difco Laboratories). Mice received 300 ng of pertussis toxin after 2 and 48 h intravenously. Development of EAE was assessed daily according to the following criteria;

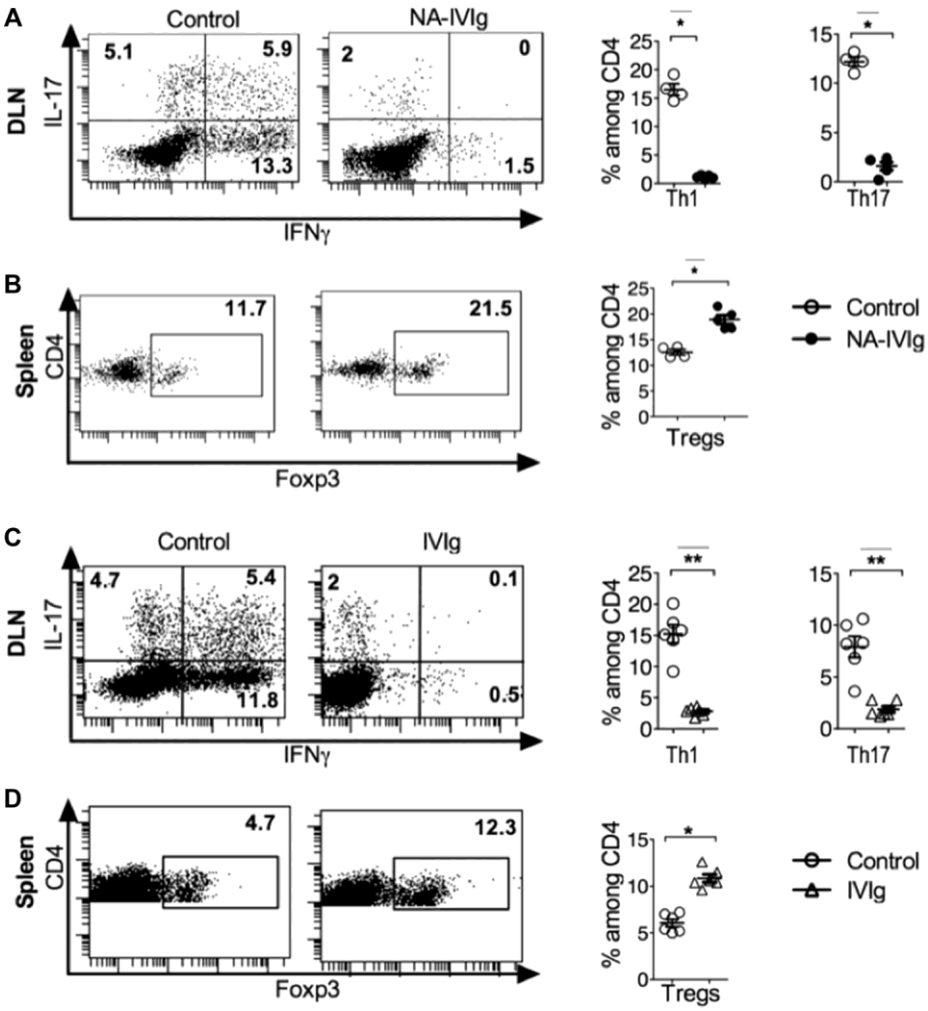

Figure 3. Neuraminidase-treated desialylated IVIg reciprocally modulates helper T lymphocytes, as does native IVIg. Nine days after EAE induction, mice were sacrificed. Spleen and draining lymph nodes (DLN) were collected and analyzed for helper T-cell subsets by flow cytometry. Representative dot plots showing $CD4^+$ T cells from (A, C) the DLN gated for IL-17 and IFN-γ; and (B, D) the spleen gated for Foxp3 in control, desialylated IVIg (NA-IVIg) and native IVIg-treated mice are shown. Value in each quadrant denotes percentage of cells positive for IL-17, IFN-γ, and Foxp3 among the $CD4^+$ population. Each symbol represents an individual mouse and data are shown as mean ± SEM from 4–6 mice pooled from two experiments (right panels, A–D). $*p < 0.05$, $**p < 0.01$, Mann–Whitney test.

0-No signs, 1-tail paresis, 2-hindlimb paresis, 3-hind limb paralysis, 4-tetraplegia, 5-moribound. IVIg (Hizentra^{§R} 20% w/v, CSL Behring) or desialylated (NA-IVIg) was given daily at 0.8 g/kg i.p from the day of the immunization until the peak of the disease (day 16–18). Control mice received equal volume PBS.

Isolation of cells from draining lymph nodes and spleen, and flow cytometry

Nine days after EAE induction, mice were sacrificed under Xylazinane/Ketamine anesthesia to obtain draining inguinal lymph nodes and spleen. Single cell suspension was prepared by mechanical disaggregation and passing through 70 μm nylon membrane filter. Red blood cells were removed using ACK lysis buffer. A total of 1.5×10^6 cells were stimulated with 25 ng of phorbol 12-myristate 13-acetate (PMA) and 1 μg ionomycin (Sigma) in the presence of Monensin (Golgistop^{§R} BD biosciences) in 10% FCS/RPMI for 4 h at 37°C. Cells were labeled with anti-mouse CD4-Pacific Blue (Clone RM4–5, BD biosciences) after blocking the Fc-receptors with anti-mouse CD16/32 antibody (BD Fc Block). Intracellular staining was performed using FoxP3 staining buffer set (eBioscience) with the following antibodies: IL-17A-A488 (clone 11B11, BD), IFN-γ-allophycocyanin (Clone XMG1.2, BD), Foxp3-PE (clone FJK16s, eBioscience). BD LSR II with FACS Diva software was used to acquire and analyze the data.

Statistical analysis

Two-way analysis of variance (ANOVA) with Bonferroni's post test was used to compare daily clinical score. Mann–Whitney's U test was used to compare parameters between control and NA-IVIg group. Values of p obtained are indicated in Figure legends.

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Abbreviations: **IVIg**: intravenous immunoglobulin · **NA-IVIg**: neuraminidase-treated IgG

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Dissecting the Anti-Inflammatory Effects of *Viscum album*: Inhibition of Cytokine-Induced Expression of Cyclo-Oxygenase-2 and Secretion of Prostaglandin E2

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Abstract

Reports unraveling the beneficial effects of *Viscum album* (VA) preparations as complementary therapies for cancer have increasingly revealed the underlying molecular and cellular mechanisms, which encompass cytotoxic properties, induction of apoptosis, inhibition of angiogenesis and several other immunomodulatory mechanisms. In addition to their propitious relevance to cancer therapy, VA preparations are also relevant for the treatment of several inflammatory pathologies. In view of the intricate association of inflammation and cancer and the fact that several anti-tumor phytotherapeutics exert potent anti-inflammatory effects, we believe that an anti-inflammatory effect is responsible for the therapeutic benefits of VA preparations. One of the underlying molecular mechanisms of this inflammatory response is the selective down-regulation of the cyclo-oxygenase (COX)-2-mediated cytokine-induced secretion of prostaglandin E2 (PGE2). This inhibitory action has been associated with reduced expression of COX-2, without modulating COX-1 expression. This mechanism is associated with VA-induced destabilization of COX-2 mRNA, thereby depleting the functional COX-2 mRNA available for protein synthesis and for subsequent induction of secretion of PGE2. Together, these results demonstrate a novel anti-inflammatory mechanism of action of VA preparation, wherein VAQUSpez an anti-inflammatory effect by inhibiting cytokine-induced PGE2 via selective inhibition of COX-2 and destabilization of COX-2 mRNA.

Viscum album Preparations

Viscum album (VA) is extensively used as a complementary therapy for cancer and also in the treatment of several inflammatory pathologies [1]. VA preparations are standardized aqueous extracts of *Viscum album L.* (commonly known as European mistletoe, a semi-parasite that grows on different host trees), composed of mainly mistletoe lectins and viscotoxins [2, 3] and several other biologically active molecules, like flavonoids, several enzymes, peptides (such as viscumamide), amino acids, thiols, amines, polysaccharides, cyclitoles, lipids, phytosterols, triterpenes, phenylpropanes and minerals [4]. Currently, at least three VA preparations based on the host tree and the method of extraction are available for therapeutic application, including VA Qu Spez (oak tree), VA P (pini) and VA M (mali).

VA preparations have been used as a complementary therapy for several types of cancer, mainly in Europe and also, to some extent, in other parts of the world [1, 5]. The therapeutic benefits of VA preparations when utilized along with surgery, chemotherapy or radiotherapy contribute to the overall improvement of the quality of life of cancer patients [6, 7]. In addition, VA preparations have been implicated as conventional phytotherapeutics in the treatment of several conditions associated with nervous system abnormalities, allergic reactions, and immuno-inflammatory disorders [8–10].

The therapeutic benefits of VA preparations in diverse pathologies have been attributed to the method of preparation, the proportion of various bioactive compounds present within the extract and the host tree. In spite of its extensive use, the precise mechanisms associated with the anti-tumoral effects of VA are not yet clear. Accumulating evidence has revealed that these preparations exert anti-tumor activities that involve cytotoxic properties [11, 12], induction of apoptosis [13], inhibition of angiogenesis [14] and several other immunomodulatory and anti-inflammatory mechanisms [15, 16]. These properties collectively define the mechanistic basis for the therapeutic benefit of VA preparations, providing strong support for their application as complementary therapies in cancer. VA preparations also exert several immunostimulatory activities by interacting with the cellular and humoral compartments of the immune system, resulting in potent anti-tumor immune responses [16–19].

In view of the therapeutic benefits of VA preparations in diverse pathological situations, including inflammation and cancer, dissecting their molecular mechanisms would contribute enormously to the understanding of the roles of phytotherapy-based treatment strategies either in complementary and alternative medicine or in other combinational therapies. The modes of action of VA preparations underlying their therapeutic benefits in inflammatory pathologies are yet to be explored. However, the successful utilization of these preparations in the treatment of certain inflammatory pathologies raises several questions related to their mechanisms of action. In this chapter, we will discuss the anti-inflammatory mechanisms of VA, with a focus on inflammatory pathways.

Inflammation and Cyclo-Oxygenase-2

Inflammation is a physiopathological symptom of infection, autoimmunity or cancer. It is a basal physiological phenomenon that comprises a complex set of responses to tissue injury or to an infectious agent to eliminate the causative agent and to initiate the healing process. The interactions of innate and adaptive immune cells as well as nonimmune cells, such as endothelial cells and fibroblasts, with inflammatory stimuli induce the production of several molecular mediators [20–22]. These inflammatory mediators act on various target tissues and exert changes in the tissues' homeostatic functions. Thus, inflammation has to be regulated, which can be achieved by various anti-inflammatory agents, such as steroids, nonsteroidal anti-inflammatory agents (NSAIDs) [23], intravenous immunoglobulins [24], immunosuppressor cells [25], and neutralizing monoclonal antibodies to inflammatory cytokines [26].

Cyclo-oxygenases (COXs) are the regulatory enzymes of the prostaglandin E2 (PGE2) biosynthetic pathway that catalyze the rate-limiting step. They convert the free arachidonic acid in the cellular cytosol obtained upon degradation of membrane phospholipids into prostaglandin H2, an active precursor for the synthesis of various prostanoids. Among the isoforms of COXs, COX-1 is constitutively expressed in cells, whereas COX-2 is induced in response to inflammatory stimuli and significantly contributes to the induction of PGE2 [27]. PGE2 is a molecular mediator of several homeostatic functions, including those of the gastric mucosa and vascular endothelium [28]. However, it also exerts potent pro-inflammatory effects, such as the induction of fever and pain. Overproduction of PGE2 occurs in response to pro-inflammatory stimuli and correlates with the severity of certain infectious and inflammatory conditions [29, 30]. PGE2 exerts autocrine and paracrine actions on target cells and can induce pro-inflammatory reactions.

The expression pattern of COX-1 and COX-2 further regulate their differential functions. COX-1 is constitutively and stably expressed at low levels in many tissues, ensuring the constant production of prostaglandins, which are essentially required for the maintenance of important physiological functions, such as platelet aggregation, normal renal functions and gastric mucosal protection. In contrast, COX-2 is typically quiescent, but its expression can be induced in response to diverse pro-inflammatory and pathogenic stimuli. When stimulated, its expression is high and transient, which leads to a burst of prostaglandin production in a regulated and time-limited manner. Thus, depending on the COX isoform, the production of the same precursor prostaglandin H2 from arachidonic acid differs with respect to the amount and timing of production. This activity can be differentially decoded by the cells, thereby leading to the activation of various intracellular pathways involving specific classes of prostaglandins and therefore different responses [31].

Because COX-2 expression is up-regulated in several pathological conditions and human malignancies, strategies for controlling the expression and activity of COX-2 have been developed as potent anti-tumor and anti-inflammatory treatments [32]. In line with the therapeutic benefits of NSAIDs, which are synthetically designed main-

ly to inhibit the enzymatic activity of COX-2, a diverse spectrum of therapeutics of natural origin, such as phytotherapeutics, has been characterized to evaluate their potential to inhibit COX-2 functioning, thereby down-regulating the pathological level of prostaglandins. Due to the structural homology of COX-1 and COX-2, most NSAIDs inhibit both of these enzymes and therefore result in several severe side effects due to the inhibition of physiological prostaglandins. Therefore, selective inhibitors of COX-2 are of great interest. Although a promising class of synthetic COX-2-selective inhibitors called coxibs has been developed, their therapeutic efficacies are compromised due to various side effects [33]. Interestingly, clinical studies have revealed the selectivity of certain plant-derived molecules in inhibiting COX-2 that are as efficient as synthetic COX-2-specific antagonists (rofecoxib and celecoxib) in ameliorating both acute and chronic inflammatory conditions [34, 35].

Role of *Viscum album* in Inhibition of Cyclo-Oxygenase-2

The long-term side effects of NSAIDs in various pathological conditions and the increasing body of evidence demonstrating the anti-inflammatory activity of plant-derived molecules together encourage the concept of the use of phytotherapeutics as potent alternatives to classical anti-inflammatory drugs [34, 36]. With growing interest in promising new-generation anti-inflammatory therapeutics, studies exploring and characterizing novel phytotherapeutics with strong selectivity for COX-2 are of great value.

With the aim of understanding the role of VA in modulating the immuno-inflammatory response, our group analyzed the effects of VA preparation on the PGE2 axis and its regulation at the level of COX [37, 38]. Our results demonstrated that one of the anti-inflammatory functions of VA occurs through the inhibition of IL-1β-induced PGE2 biosynthesis. The expression pattern of cytokine-induced COX-2 detected in the presence of VA preparations confirmed that IL-1β induces the expression of COX-2 mRNA, while VA does not. In contrast, VA significantly inhibited the COX-2 protein expression induced by IL-1β. These results suggest that VA exerts a post-transcriptional regulatory effect on COX-2 expression [39].

However, the effect of VA preparation is not solely restricted to IL-1β-induced COX-2 expression. VA could also inhibit IFN-γ- and TNF-α-induced COX-2 expression (unpublished data), indicating that the suppression of COX-2 by VA occurred in response to inflammation induced by a wide range of cytokines. The fact that at the later phases of cytokine induction, VA did not inhibit COX-2 suggests that inhibition of COX-2 by VA occurs in the early phase of COX-2 regulation, but not in the later phases. Further, at each time point, the treatment of cells with VA resulted in reduced expression of IL-1β induced COX-2.

There was no significant difference in the degradation pattern of COX-2 in cytokine-stimulated cells with or without VA treatment. Additionally, VA induced destabilization of COX-2 mRNA, thereby diminishing the functional mRNA available for

protein synthesis and for subsequent induction of secretion of PGE2 [38]. Thus, the modulation of COX-2 by VA strongly supports its value as an anti-inflammatory therapeutic.

Concluding Remarks

The prolonged administration of anti-inflammatory COX-2 inhibitors has been ineffectual for chemopreventive and chemotherapeutic purposes since the risks prevail over the benefits. The clinical demonstration of severe side effects due to the failure of classical COX-2 inhibitors to discriminate between aberrant pathological and homeostatic functional activation states has raised the concern that direct COX-2 enzymatic inhibition might not represent a sufficiently appropriate clinical strategy to target COX-2. Several phytotherapeutics have been shown to exert modulatory effects on COX-2 at various levels of its molecular regulation and therefore have been considered as effective alternative strategies for controlling the pathogenic expression of COX-2.

Given that VA preparation exerts potent anti-inflammatory effects by selective down-regulation of COX-2, it is extremely interesting to dissect the COX-2 inhibition mediated by VA in association with different regulatory mechanisms at the molecular level. VA exerts anti-inflammatory effects by interfering with cytokine-induced PGE2 biosynthesis through selective inhibition of the COX-2 protein, suggesting a beneficial role with minimal side effects. These observations are relevant to understand the mechanisms of action of VA preparations and may provide insights for the further exploration of their anti-inflammatory mechanisms in diverse pathologies. Interestingly, in these studies, no changes in the expression of COX-1 were observed at any of the concentrations of VA preparation that inhibited COX-2, irrespective of robust stimulation by IL-1β. These findings suggest a strong selectivity in the anti-inflammatory mechanisms of VA preparation in inhibiting COX-2 expression. Given that VA exerts potent anti-inflammatory effects by the selective down-regulation of COX-2, it is extremely interesting to dissect the COX-2 inhibition mediated by VA in association with different regulatory mechanisms at the molecular level. Increasing evidence for the anti-inflammatory mechanisms of action of these preparations will lead to greater acceptance of their use for the treatment of various pathologies.

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